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MECHANISM OF MICROBIOLOGICAL CONTAMINATION OF JET FUEL AND DEVELOPMENT OF TECHNIQUES FOR DETECTION OF MICROBIOLOGICAL CONTAMINATION

Gordon C. Blanchard

Third Quarterly Progress Report

1 September 1963 to 1 December 1963

AF Aero Propulsion Laboratory
Research and Technology Division
Air Force Systems Command
Wright-Patterson Air Force Base, Ohio

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FOREWORD

This is the third quarterly report prepared under Contract AF 33(657)-9186 "Mechanism of Microbiological Contamination of Jet Fuels and Development of Techniques for Detection of Microbiological Contamination." This contract was initiated by the Aero Propulsion Laboratory, Research and Technology Division, Wright-Patterson Air Force Base. Mr. A. Churchill is Project Engineer.

This report concerns work done from 1 September 1963 to 1 December 1963.

ABSTRACT

This study is concerned with the detection of microorganisms in fuel water-environments and with the mechanisms by which these microorganisms cause problems in fuel systems of aircraft. The laboratory detection methods studied were the esterase approach, tetrazolium reduction, O_2 consumption and CO_2 evolution. The techniques which appear to have the greatest chance of success are the esterase approach and tetrazolium reduction. For field detection, the work is continuing on the electrophoresis-pulse polarization technique. These studies have been extremely successful in finding techniques which, by the end of the year, should be useful for rapidly detecting small numbers of viable microorganisms in fuel water bottoms. The esterase technique now can detect 10^6-10^7 viable cells in about 5 minutes and it is expected that the tetrazolium technique will determine the number of organisms which can utilize fuel. All that remains to be done now is to establish the criteria, such as the length of time required for color to develop, which will indicate when the microbial contamination has reached a harmful level.

The field detection approach has been confined to the calibration of the pulse polarization technique using solutions in which aluminum corrosion is known to occur. The results demonstrate that there are three polarization characteristics - double layer capacitance, exchange current and tafel slope - which affect anodic and cathodic polarization. The results to date appear to be promising, because alteration in any one of these 6 characteristics by fuel microorganisms could be used as a means of detection.

The metabolism study is concerned primarily with defining the corrosion problem and in defining the chemical changes which occur during the growth of

microorganisms. The role of microorganisms in corrosion has not been established, but considerable progress was made during this quarter in defining the problem. The results show that the mineral content of the growth medium plays an important role in corrosion. Anions, such as nitrate and sulfate, and cations are important; Mg-nitrate corrodes aluminum slower than distilled water, while Mg sulfate corrodes aluminum faster than distilled water. The results suggest that microbial corrosion is inhibited by the metal constituents in the present growth medium. That is, the metal cofactors required by the enzymes responsible for oxidation of hydrocarbons are supplied, and therefore they are not removed from the alloy by the organism. An additional possibility is that microbial corrosion in fuel systems may be enhanced by the microbial deterioration of materials used to protect the aluminum from corrosion. The results may show that both mechanisms are involved. These hypotheses will be tested in future studies.

The changes in the composition of fuel and water layers produced by microorganisms during growth were investigated. The changes in IR absorption of fuel before and after growth were characterized. The fuel soluble compounds produced during growth appear to be polynuclear hydrocarbons; no oxygenated compounds were observed by IR analysis. In contrast, several of the emulsion forming pure culture isolates were shown to produce water soluble oxygenated compounds during growth. Further study of these oxygenated compounds in relation to the corrosion study is planned.

The effect of growth on the O_2 and CO_2 levels of the fuel and the water phases of the growth medium were followed in open and closed systems. In closed systems the organisms utilized the O_2 and produced CO_2 in the media.

The effect of these changes on ecological changes in microflora will be examined once conditions for demonstrating corrosion by microorganisms are established.

The permeability to fuel of fuel and normal cultures was studied but the problem has not yet been resolved.

Further studies with deionized water and distilled water are planned to explain the growth of fuel isolates which occurred in the BH-medium lacking nitrate. In order to elucidate the growth of fuel isolates on BH-medium lacking nitrate, the metabolism of these organisms in distilled or deionized water will be studied.

A preliminary comparison of some new mineral media indicated that better growth was obtained with the new media than with BH. The corrosivity of this medium is now being compared to BH.

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I. INTRODUCTION

The growth of microorganisms in hydrocarbon substrates, especially fuels, has been noted for many years. In some cases the organisms are associated with difficulties; i.e., corrosion of iron, the problems of water pollution and waste from refineries, the decomposition of additives in gasoline, the formation of water-oil interface of microbial masses which clog fuel lines, and corrosion of aluminum in the wing (integral) fuel tanks of aircraft. The direct involvement of microbes in these problems and especially the mechanisms by which the various difficulties are brought about are still poorly understood. It is believed that definite answers will be obtained by the studies planned in this program.

Rapid detection techniques, mechanisms of corrosion production, and changes during growth of fuel organisms were investigated during this quarter. The rapid detection approaches considered were esterase, tetrazolium reduction, O_2 consumption, CO_2 evolution and pulse polarization. In the mechanism study, the role of microorganisms in corrosion and their role

in the utilization or production of compounds in fuel-water environments was investigated.

Two different detection approaches are needed; the first four methods are possible approaches for detecting changes in the concentration of microorganisms in laboratory experiments. The last approach, pulse polarization, is a possible method for the detection of corrosion organisms in the field before they cause subsequent deterioration of fuel systems. In the mechanism study, laboratory information is needed which will help define the problems associated with the growth of microorganisms in fuel. During this quarter experiments were set up to define the role of microbes in corrosion, and to determine what changes occurred during growth of the microorganisms in the fuel-water environment. The changes of environment during growth, (i.e., CO_2 and O_2 content of the medium and in the ultraviolet and infrared absorption of the fuel and water layers) were investigated.

Two other studies were continued: the permeability study and the nutritional study. The effect of fuel on the viability of organisms was investigated in the hope of determining whether fuel organisms are less permeable to fuel than normal cells. Preliminary studies with new media were initiated to determine if such media are less corrosive than the BH-medium and if differences in corrosion could be associated with the increased growth of fuel organisms.

II. SUMMARY AND CONCLUSIONS

A. Detection Studies

Since the beginning of this program, several rapid detection approaches have been evaluated for their possible application to the quantitation of microbial numbers in laboratory fuel studies. The approaches evaluated to date are: the use of microbial esterases, gas chromatography of lipids, indicator soda lime technique for absorption of evolved CO_2, gas chromatographic measurement of evolved CO_2, tetrazolium reduction, and oxygen consumption. Only two of the techniques evaluated, the esterase and tetrazolium reduction, appear to warrant further consideration. The other techniques were considered inappropriate because they lacked the sensitivity required, were not rapid enough, or failed to give a measurable response with jet fuel organisms.

Four techniques were evaluated during this quarter including esterase, tetrazolium reduction, oxygen consumption and CO_2 evolution. The results of experiments with the esterase approach showed that the esterase level of the organisms does not increase by the same increment as the cell count. In rich media, TGY and lactate, the enzyme activity was related to the growth phase but in fuel media this relationship was not apparent. It was found that the enzyme level is associated with the energy source of the medium, and that it is about 10 fold higher in TGY than in lactate and about 10 fold higher in lactate than in BH-fuel. The same general relationship was observed with cell numbers. The TGY medium contained approximately 10^{10} cells/ml at the cessation of growth, whereas lactate and BH-fuel has about 10^9 and 10^8 cells/ml respectively.

The tetrazolium reduction approach for detecting fuel organisms appears to be most useful. Only preliminary studies have been carried out to date and no relationship between activity and cell numbers has been made. However, all of the cultures examined to date reduced the dye vigorously.

The conditions for assaying the dehydrogenase with tetrazolium in fuel isolates have been determined and the studies concerning the role of various substrates and cofactors in this reduction have been worked out. Preliminary results indicate that in the presence of MTT tetrazolium glyceraldehyde-3-phosphate, DPN and fuel microbes in BH_4 it is possible to easily detect the organisms in 30 minutes. The objective is to reduce this time to 5 minutes, and in addition, show the relationship between cell numbers and tetrazolium reduction and the changes in tetrazolium reduction during various phases of growth.

The tetrazolium technique is particularly good because upon reduction the dye forms a precipitate insoluble in water. The precipitate is soluble in fuel and it is easily extracted from the water phase into the fuel phase by shaking. Thus, it is possible to measure the color developed in fuel and obtain a quantitative readout of the enzyme (dehydrogenase) activity of the fuel culture. Further studies along these lines will be carried out in the next quarter.

We have been fortunate in finding two approaches that will be useful in monitoring the levels of microbial contamination of fuel. It is anticipated that criteria for determining the critical harmful levels of organisms will be defined in future months and that it will be possible to detect the level of microbial contamination by color changes in fuel water systems containing a small amount of either esterase or tetrazolium reagents.

Work in the field detection approach has been confined to the calibration of the pulse polarization technique using solutions in which aluminum corrosion is known to occur. The results demonstrate that there are three polarization characteristics - double layer capacitance, exchange current- and tafel slope, which affect anodic and cathodic polarization.

The results achieved appear promising because alteration in any one of these 6 characteristics by fuel microorganisms could be used as a means of detection.

B. Metabolism Studies

During the past three quarters the corrosion of aluminum alloys by microorganisms has been investigated at the cellular level. Attempts were made to design definitive experiments capable of showing the extent to which microorganisms contribute to the corrosion process.

Unambiguous results were obtained initially. Media capable of supporting the growth of fuel isolates did not themselves cause corrosion in the absence of microbial growth. However, when these media were inoculated with fuel isolates, corrosion of aluminum alloys was apparent.

In an attempt to verify and to further investigate the relationship of microbial growth to corrosion processes, a still larger number of observations were made. During these studies it was apparent that, with large numbers of controls, a certain incidence of corrosion occurred in the absence of bacterial growth. This observation suggests a possible lack of uniformity in the structure of the alloy under investigation, and points up the limitations of the initial preliminary experiments which utilized fewer controls.

In the third quarter, the effects of growth media constituents has received additional attention and the observation was confirmed that the modified BH medium causes corrosion without the addition of bacteria.

The design of a medium capable of supporting the growth of fuel isolates, but which does not corrode aluminum alloy is a formidable task, and it is a problem which at the present time has not been solved by any investigator concerned with corrosion problems.

Two phenomena relating to the involvement of microbial growth in jet fuel systems deserve consideration primarily because they suggest distinct modes by which microbial growth may be related to corrosion occurrence.

First, the monomethyl ether of ethylene glycol is known to inhibit or prevent fuel tank corrosion when used as an additive to conventional fuel systems. This compound has a known bacteriostatic or bacteriocidal action. The ability of the ether to prevent corrosion is therefore presumptive evidence that microorganisms are intimately, if not causally, involved in fuel tank deterioration. But the use of the monomethyl ether of ethylene glycol for corrosion prevention does not reveal the chemical mechanism of bacterial attack.

This ester may cause the lysis of bacteria and the dilution of their enzymic constituents, or the compound may act only as a competitive or noncompetitive inhibitor of enzymes essential to the metabolism of fuel in the corrosion process. In the latter case, it is easily conceived that prevention of corrosion would not alone alleviate difficulties deriving from foam formation, or the possible insensitization of instrumentation by biogenic sludge.

Secondly, during the course of the research effort, it was found that nitrate ion prevented corrosion of aluminum alloys in the unmodified BH medium. When NO_3^- was removed from the BH medium the ions present caused considerable corrosion in the absence of microbes. At the present time this observation has not been rationalized in terms of the chemistry of the inorganic reactants involved. However, the ability of a given reactant to prevent corrosion suggested the hypothesis that bacterial growth may remove substances which inhibit corrosion, in this case the nitrate ion.

In the medium employed, the removal of nitrate is a concomitant of cell growth, while the Ca^{++}, Mg^{++}, and Fe^{+++} of the medium or their anionic salts, appear to catalyze the corrosion of aluminum alloys in the absence of nitrate. In this context the growth of microbes in fuel indicates the degree of contamination of fuel systems by quantities of minerals which support growth and at least one of which inhibits corrosion formation.

Should analysis indicate that fuel or water bottoms contain minerals in sufficient concentration to support growth of fuel isolates, then the suitability of treating fuels with ion exchange resins or chelating agents, soluble in both organic and aqueous phase, would require consideration. Attention would be directed again to the physiological character of the fuel isolates, with particular reference to their growth requirements. The latter consideration is an important aspect of the study dealing with methods for detecting fuel organisms.

Based on the data and conclusions that have been obtained, it appears that the corrosion problem is becoming more defined. There are three hypotheses concerning the mechanism by which microorganisms cause corrosion. The first hypothesis is that fuels contain minerals that support bacterial growth, and that it is the alteration in this mineral content effected by bacteria, which specifically produce an ionic environment causing corrosion.

Following evaporation of fuel, possible residues will be examined for metals which (1) catalyze corrosion of aluminum, (2) inhibit corrosion, and (3) inhibit or support the growth or metabolism of fuel isolates.

If materials are obtained from fuel which support growth and affect metal surfaces, such as Fe^{+++} has been shown to do, then the fixation of

these components by fuel organisms will be explored with the objective of assessing the ecological changes in a fuel-water system having some consequence both in the problem of corrosion and the problem of biological sludge formation.

The second hypothesis is that microbial growth in fuel tanks is possible because organisms remove metals from the alloy surface for growth. Two mechanisms are suggested to account for this activity, (1) the elaboration by microorganism of specific compounds which react with metals and render them soluble, and also make them capable of penetrating the bacterial cell, and (2) the ability of the microbes to shift the equilibrium as follows:

Metal - alloy $\rightleftharpoons$ Metal - water $\rightleftharpoons$ Metal - microbe in the direction of the microbial cell.

There is some recent evidence by Gholson, Baptist and Coon[1] which shows that aluminum ions will substitute for ferrous and ferric ions in one of the enzyme systems associated with the conversion of octane to octanol. This is an important finding because it suggests that in the absence of any other cations such as Fe^{++}, Mg^{++}, Ca^{++} etc. that microorganisms may remove the aluminum oxide from the surface of the aluminum and thus accelerate or initiate corrosion. Pure cultures are now growing, in the laboratory, in pure hydrocarbon systems and forming emulsion type materials. The emulsion will be analyzed by infrared and gas chromatography to determine the composition of the materials. The next experiments will involve the production of these compounds in a medium with limited nutrients in the presence of aluminum alloys so that it may show the relationship of emulsion to corrosion.

An alternate hypothesis is suggested by the work of Hamilton et. al.[2] who demonstrated hydrocarbon oxidation in the absence of enzyme. It may be that bacteria produce compounds such as ascorbic acid which will react with the metal surface and cause corrosion in the presence of oxygen. If this hypothesis is true, it may not be sufficient just to kill the organisms to eliminate corrosion, but one may also have to remove the organic materials. This possibility will be examined.

The third hypothesis is that microbes are utilizing the sealants and topcoats and possibly the corrosion inhibitors in the fuel systems so that the aluminum alloys are exposed to the normal electrochemical corrosion.

In those situations where aluminum tanks are coated with plastic sealants the operation of the first mechanism could be of special importance because of the possible trapping of organisms on the metal surface. The trapped organism may give rise to a colony of bacteria, and because of the confinement any metabolic product would exist in extremely high concentration. Such products known to affect metals may be no more complex than the hydrogen ion or citric acid, a chelate, which forms compounds of great stability with a wide variety of metals.

In the absence of microbial growth, model systems using various chelating agents can be designed which reveal the consequence with respect to corrosion of selectively removing the constituent metals of an aluminum alloy. The effect of corrosive stimulants, such as ferric ion and magnesium, can be studied in a similar manner, and the concentration levels at which they are effective can be determined.

Experiments will be conducted during the next quarter to determine which of the three hypothesis concerning corrosion is most important. The possibility exists that it is not any one mechanism alone which causes corrosion but a combination of one or more mechanisms which change as the conditions are varied.

Ultraviolet and infrared spectroscopy and fluorescence measurements were made of fuel and water phases of growth media before the initiation of growth and following its cessation. These analyses were made to detect the utilization of fuel components during growth and the production of water soluble or fuel soluble compounds singularly characteristic of fuel-grown organisms. Knowledge of such compounds would be useful in explaining the metabolic pathways of fuel organisms, as well as being useful in this identification.

The growth of microorganisms in a fuel-water environment caused the production of compounds which were soluble in the fuel as demonstrated by increased ultraviolet absorption. Analysis of these compounds by ultraviolet and infrared absorption, and by fluorescence indicated that low concentrations of these compounds, possibly polynuclear hydrocarbons or conjugated unsaturated compounds, were present. The preliminary infrared analysis indicated that these compounds might be oxygenated. However, fractionation by distillation failed to confirm this expectation. Further work with these complex compounds will be considered with pure hydrocarbons in the growth medium.

One interesting observation found in this quarter was that the ultraviolet absorption characteristics of fuel dissolved in water. A peak at 271 mμ was observed which disappeared when fuel microorganisms were allowed to grow only on the fuel that was soluble in the water. Whether this component is the only one utilized is not known, but it is doubtful. Attempts to determine this will be considered in future work. The growth of microbes in fuel-water

environments, however, do produce oxygenated compounds. This was demonstrated by use of a pure culture of an emulsion forming organism grown on JP-fuel media. Infrared analysis showed that some oxygenated component, possibly acid, alcohol, ketone or aldehyde was formed during growth.

Work is now in progress to produce these water soluble emulsion type compounds from pure hydrocarbons. The relation that such compounds have to the corrosion and sludge formation problems are also under consideration.

After characterizing these compounds, it is projected that a "model system" can be built which will demonstrate corrosion when the indicated compounds are formed.

With the object of understanding the metabolism of those organisms which grow in fuel water systems and which are to be detected, analyses were made of the removal of oxygen and the production of CO_2 by them. The oxygen electrode was employed to determine oxygen uptake and a titration method was used for CO_2 measurements. The observation was made that oxygen was consumed and CO_2 was evolved in a closed system. The intermediate oxidation products formed during this 2 to 3 day incubation period were not studied.

The tentative hypothesis was tested that fuel organisms differed from normal organisms with respect to fuel permeability, and further that this difference would result in the survival of organisms in water-fuel systems. Observations were made which suggested that salt concentration and previous growth conditions profoundly affect the viability of both normal and fuel organisms when exposed to fuel. These observations indicated that fuel organisms can be made sensitive to lethal effects of the fuel, which under other conditions serves as an energy source for these organisms.

Fuel organisms grow slowly when using hydrocarbons as substrates. The slowness of growth impedes both the analysis of their metabolic processes and the analysis of the relationship of such growth to corrosion processes. Therefore, nutritional studies were initiated to develop media which would simulate the character of fuel and yet permit rapid growth. In addition to the testing of media, preliminary studies were made of the effect of copper which was observed to first repress then stimulate growth.

III. FUTURE WORK PLANNED

Work for the next quarter will include a continuation of the studies conducted during the third quarter, in addition to new work.

Work to be continued will include:

A. Detection Studies. Laboratory detection techniques to be continued will include the esterase enzyme approach and tetrazolium dye studies. Attempts to quantitate these methods for laboratory and field use will be made. Field detection studies, i.e., activation polarization characteristics, will also be continued.

B. Studies of the Corrosion Mechanism. Corrosion studies during the fourth quarter will be designed to evaluate three postulated corrosion mechanisms involving bacteria. The first hypothesis, that fuels contain minerals which support bacterial growth and that the alteration in this mineral content effected by bacteria specifically produces an ionic environment causing corrosion, will be investigated by examining fuel residues for metals which (1) catalyze corrosion of aluminum, (2) inhibit corrosion, and (3) inhibit or support the growth or metabolism of fuel isolates.

The second hypothesis, that microbial growth in fuel tanks is associated with the organisms, removal of metals from the alloy surface during growth, will be investigated. The use of an emulsion forming organism and pure hydrocarbon systems will be employed for these studies. Possibly specific compounds produced during growth might solubilize the metals and allow their penetration into the bacterial cell, as well as critically alter the metal surface with respect to corrosion susceptibility.

The third hypothesis, that microbes utilize sealants, topcoats, and corrosion inhibitors in the fuel, thus exposing the alloys to normal electrochemical corrosion, will also be investigated.

Because the softer 2024 alloys were more susceptible to corrosion, an examination of the contribution of hardness and structure of the alloy to amount of corrosion observed in the growth medium will be studied.

Studies of the corrosion mechanism by half-cell techniques will be continued. These studies will include investigation of corroded and non-corroded alloys in conjunction with large numbers of washed cells in deionized and distilled water.

C. Nutritional Studies. Nutritional studies will determine if corrosion occurs more rapidly in Bushnell-Haas fuel medium than new media presently under investigation.

D. Permeability Studies. Permeability studies will point out differences in fuel isolates and normal cultures.

IV. EXPERIMENTAL WORK

A. Rapid Detection Studies

The objectives of the detection study are to find a rapid detection technique which can be used as a research tool for the detection of fuel microbes in laboratory studies, and to develop a detector which will rapidly indicate the presence of low levels of contamination before they cause any deleterious effects on the environment. This means that the probe must detect minute quantities of microorganisms in a short period of time and must supply the information required to identify the quantity and type of organism present. The difficulties involved in the development of such a probe are: (1) analytical techniques of sufficient sensitivity are limited in number, and (2) most analytical techniques require long time periods. Consequently, new schemes must be developed to detect microorganisms, and signal to noise ratios must be increased. The detectors studied for laboratory use and field use are described below.

1. Laboratory Detection Techniques

During this quarter four possible techniques were investigated from the standpoint of being useful for indicating numbers of organisms in laboratory experiments. The detection approaches considered in this quarter were: the esterase enzyme, tetrazolium reduction, oxygen consumption and CO_2 evaluation. The results obtained with each of these techniques are described below.

a. Comparison of Esterase Activity of Fuel Cultures: Previous studies (Second Quarterly Report) demonstrated that the esterase level of microorganisms increased during the various phases of growth but that the increase was not proportional to the increase in cell numbers. These studies were repeated during the third quarter with Cultures 5, 14 and a kerosene water bottom culture. The cultures were grown for 72 hours at 30°C on the shake

machine on TGY, BH-fuel and lactic acid salts medium (appendix, formula F). Fifty mls of sterile JP-4 fuel were overlaid on the media for each of the cultures.

Figure 1 shows the esterase activity of the three cultures on TGY overlaid with fuel. Figure 2 represents the esterase activity of the three cultures on lactic acid medium. Figure 3 represents the esterase activity of the three cultures on BH-fuel medium.

Samples were taken for total viable counts each time esterase assays were run, but unfortunately, due to contamination of the dilution pipettes the results were lost. A generalization can be made however, based on the turbidity of the cultures. TGY was most turbid, lactate was next, and BH had the least turbidity, indicating that most growth occurred in TGY, then lactate, then BH. This observation agrees with the esterase levels in the three different media where 10 fold differences in enzyme activity were observed between each of the 3 media, with TGY being approximately 100 fold better than BH.

The one important observation from the metabolism standpoint is that fuel organisms in fuel-salts medium show a variable esterase response during growth. Nevertheless, the data obtained to date still suggest that it is possible to set-up a simple test to indicate the various levels of contamination in fuel-water samples. When more information is available about the levels of contamination associated with the various problems, especially corrosion, it will be possible to establish criteria for indicating when the harmful level of microbial growth is exceeded.

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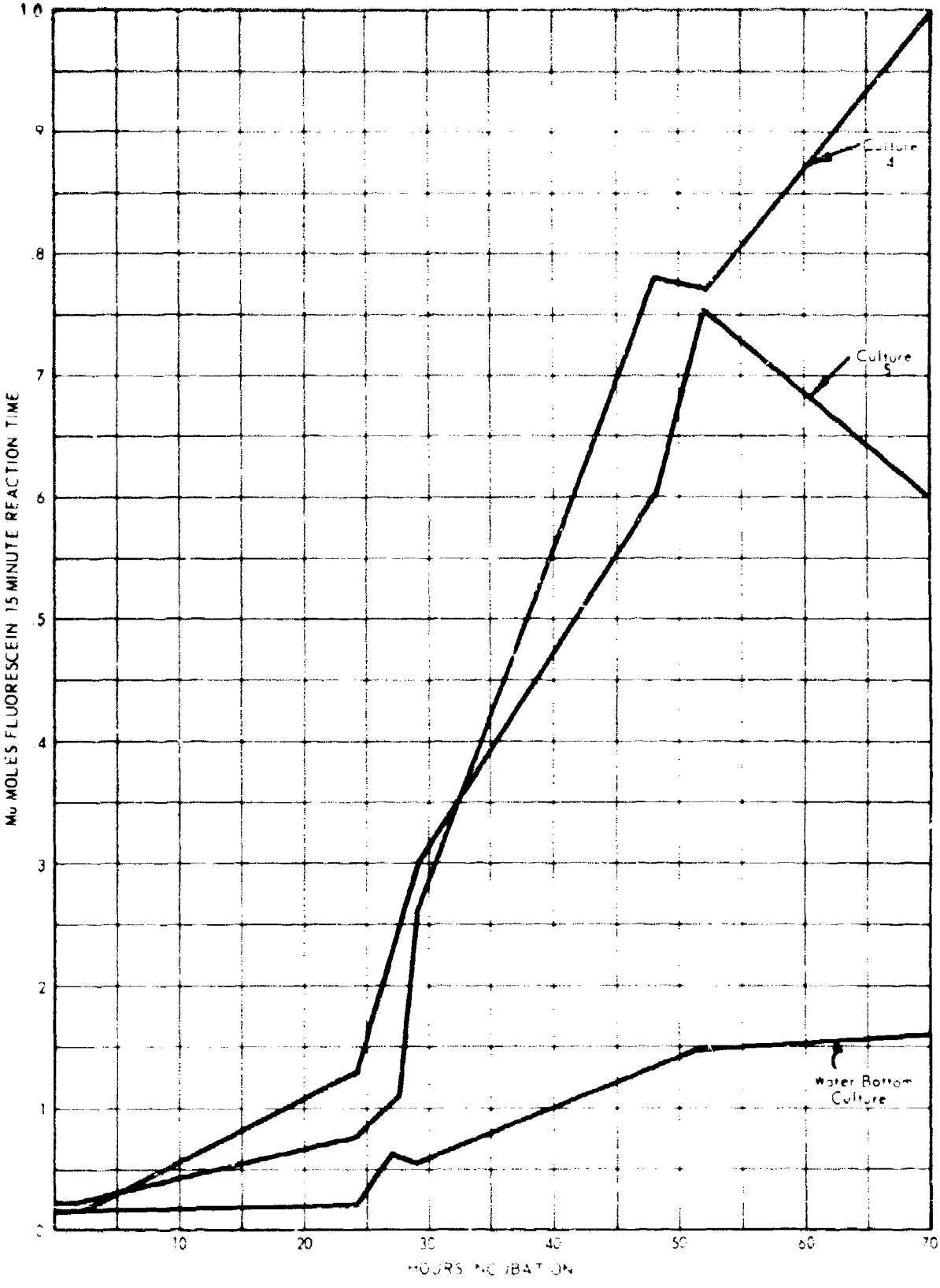

Figure 1. Esterase Activity of Fuel Organisms Growing on TGY Fuel Medium

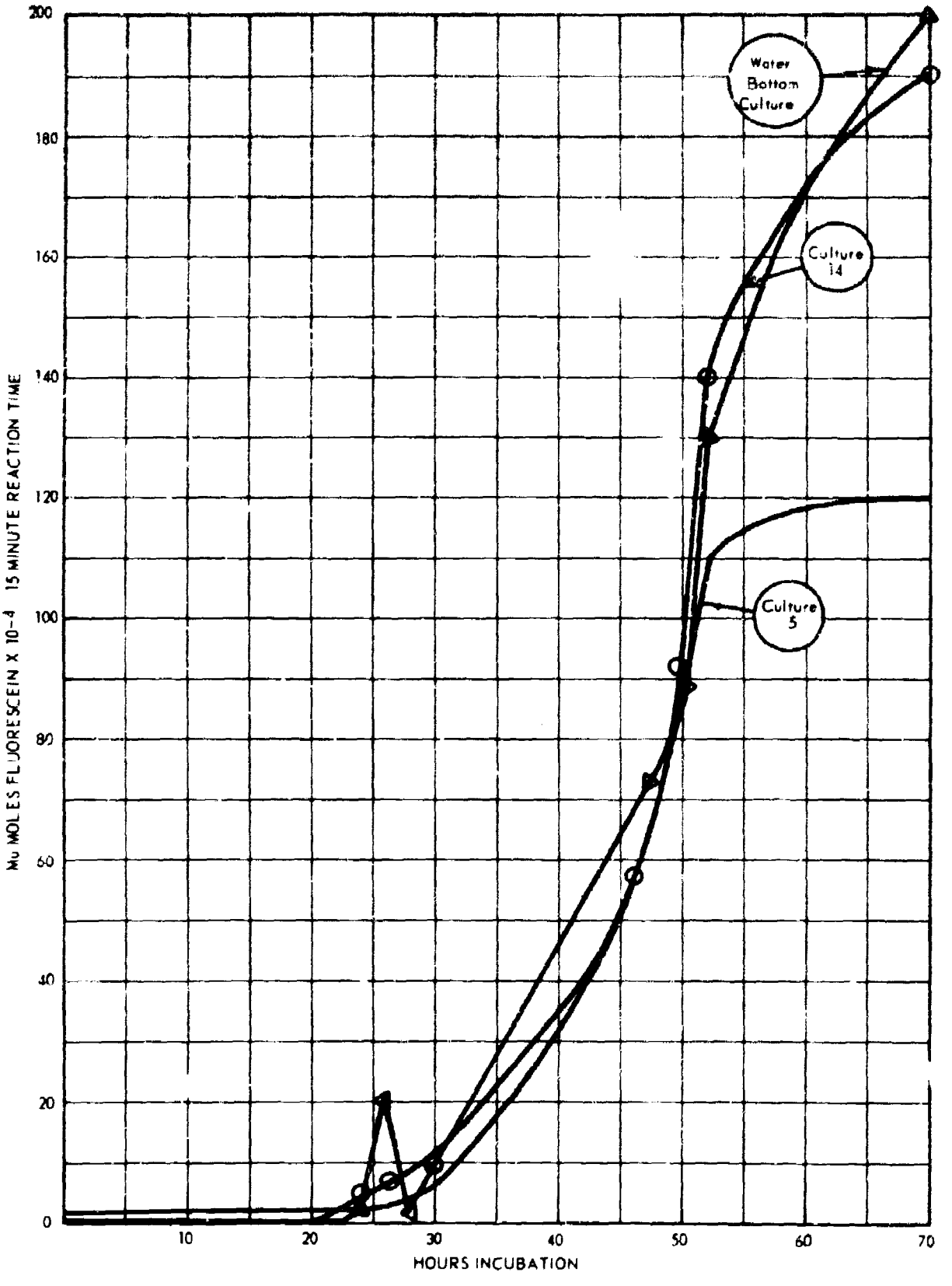

Figure 2. Esterase Activity of Fuel Organisms Growing on Formula F Lactate Fuel Medium

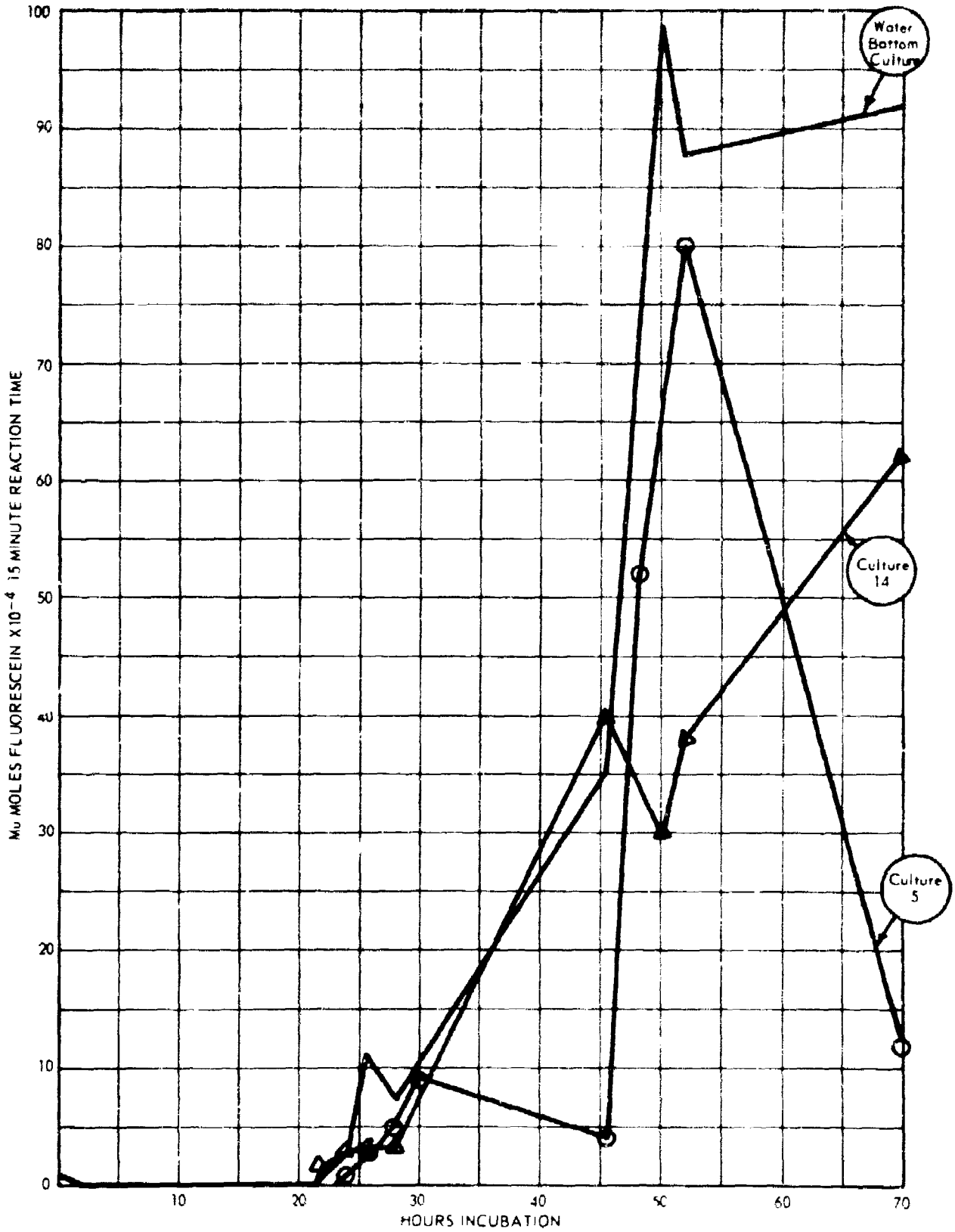

Figure 3. Esterase Activity of Fuel Organisms Growing on BH-Fuel Medium

Other esterase substrates, i.e., indoxyl acetate, will be investigated during the next quarter to determine if the esterase response observed with fluorescein diacetate is a peculiarity of the substrate. Indoxyl acetate upon hydrolysis by the organism forms indoxyl which immediately oxidizes to an insoluble dimer, indigo blue. This substrate has been employed for histochemical studies and deposits indigo blue in cells. Whether this is true with bacterial cells is now under investigation.

b. Tetrazolium Dye Study: Several tetrazolium salts have been shown to function as electron acceptors in dehydrogenase mediated reactions[3]. The reduced dye or formazan is insoluble in water, but the water insoluble formazan can be extracted into organic solvent, producing a red to violet color depending on the particular formazan used. The formazan absorbs intensely over a large part of the visible spectrum, and its absorption with respect to concentration is linear.

The enzymatic formation of insoluble formazans appeared to be ideally suited for use in the jet fuel detection problem. If bacteria growing on fuel would reduce the salt, the formazan would then be extracted into the fuel, which could be measured colorimetrically. Several experiments designed to test this hypothesis are described in the next section.

Three dyes were selected to be tested with a group of JP-4 isolates and corrosion organisms. The commercial formazans of the three dyes, and the formazans produced by chemical reduction with sodium hydrosulfite, were dissolved in JP-4 and scanned spectrophotometrically in the Zeiss PMQ-2 spectrophotometer. The dyes selected were MTT (3,4,5 Dimethyl tiazolyl 1-2) 2,5 Diphenyl tetrazolium bromide, NTV (Neotetrazolium Violet), and NTC, (Neotetrazolium chloride).

MTT, 1.0 mg per ml in H_2O, was reduced with sodium hydrosulfite, and extracted with 3.0 ml of JP-4 fuel. The extracted formazan was scanned from 400 mμ to 600 mμ. The results are shown in Figure 4. The peak although very broad, appears to lie at 570 mμ. A similar scan was run with NTV formazan, concentration 0.5 mg per ml, diluted 1:20, and extracted into 3.0 ml of JP-4. The results are also shown in Figure 4. NTC, 0.5 mg per ml was

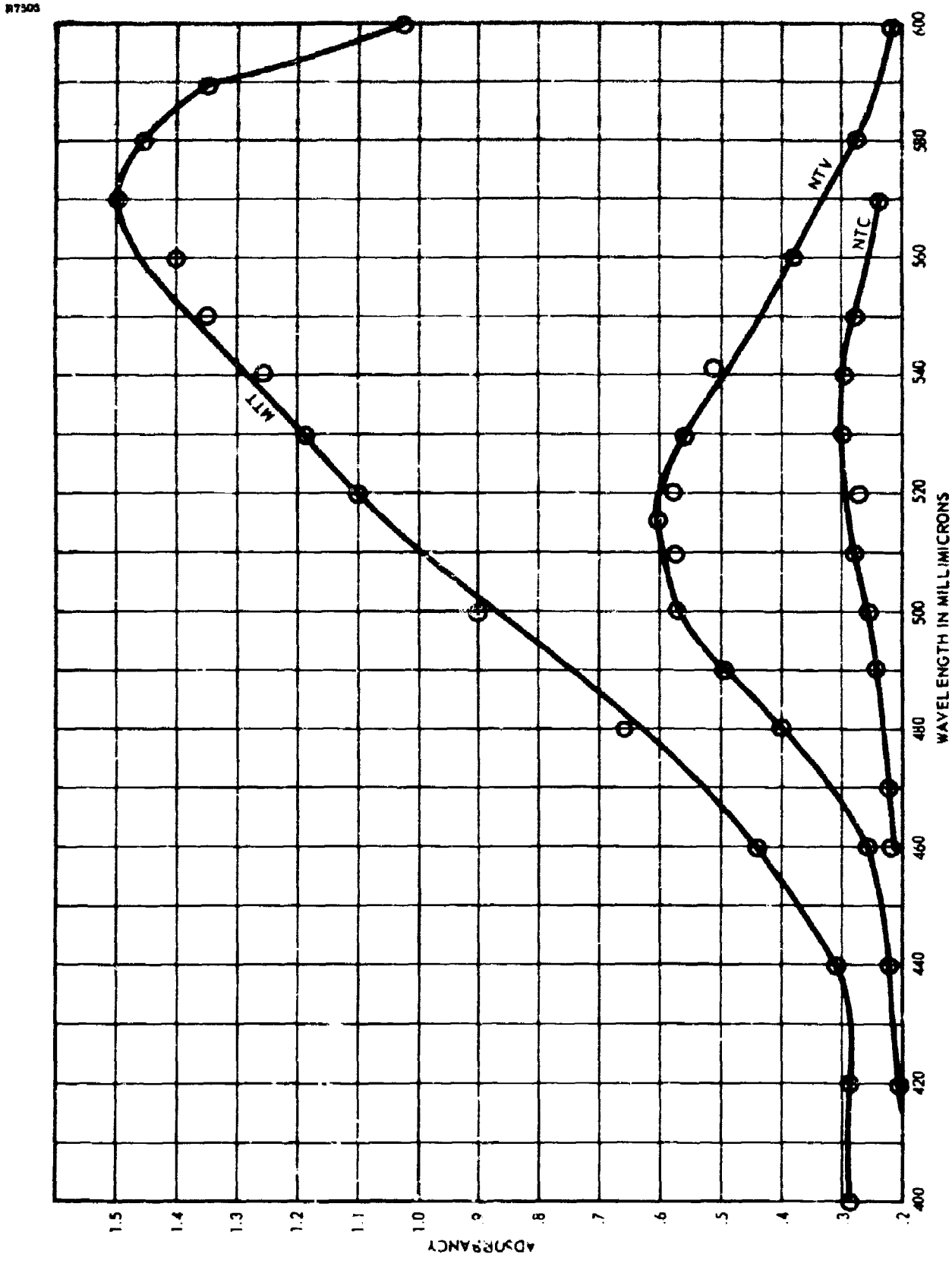

Figure 4. Adsorption of MTT, NTV, and NTC Formazans in JP-4 Fuel

extracted in the same manner into 4.0 ml of JP-4. NTC clearly showed the broadest peak (Figure 4.).

Of the three formazans tested, MTT appeared to be the most easily detectable. A standard curve of the formazan was run at 570 mμ from .005 mg to .050 mg per ml. The results are shown in Figure 5. It should be possible on the basis of this curve to detect 5 micrograms of the formazan at this wavelength.

To test the ability of the jet fuel organisms to reduce MTT, 10 corrosion organisms, and 10 fuel isolates were selected. To 3.5 ml samples of 3 week old cultures of these organisms in Bushnell-Haas salts media, 0.2 ml MTT (10 mg per ml in H_2O) was added. The bacteria -MTT mixture was overlaid with 4.0 ml of sterile JP-4 fuel, agitated for 30 seconds on a Vortex mixer, and allowed to stand for five minutes. A control containing only Bushnell-Haas and MTT was treated in the same manner. A 0.3 ml aliquot of the extracted formazan was diluted to 3.0 ml in sterile fuel, and the absorption measured at 570 mμ. The results are shown as the 0 time reading in Table 1. The tubes were placed on the rotating apparatus shown in Figure 6, and agitated at 10 rpm. Readings were made at 3 hrs, 24 hrs, and 48 hrs. The results are shown in Table 1. The rates of reduction varied considerably among the various organisms. After 3 hrs, there was reduction in 50 percent of the samples and in 24 hrs there was reduction in all but one of the samples.

Since the mechanism of tetrazolium dye reduction may be associated with dehydrogenase enzymes, DPN 10 mg per ml 0.3 ml was added to the dye. All concentrations and procedures were as described for the first series. It was observed that in the absence of added substrate, i.e., succinate, lactate

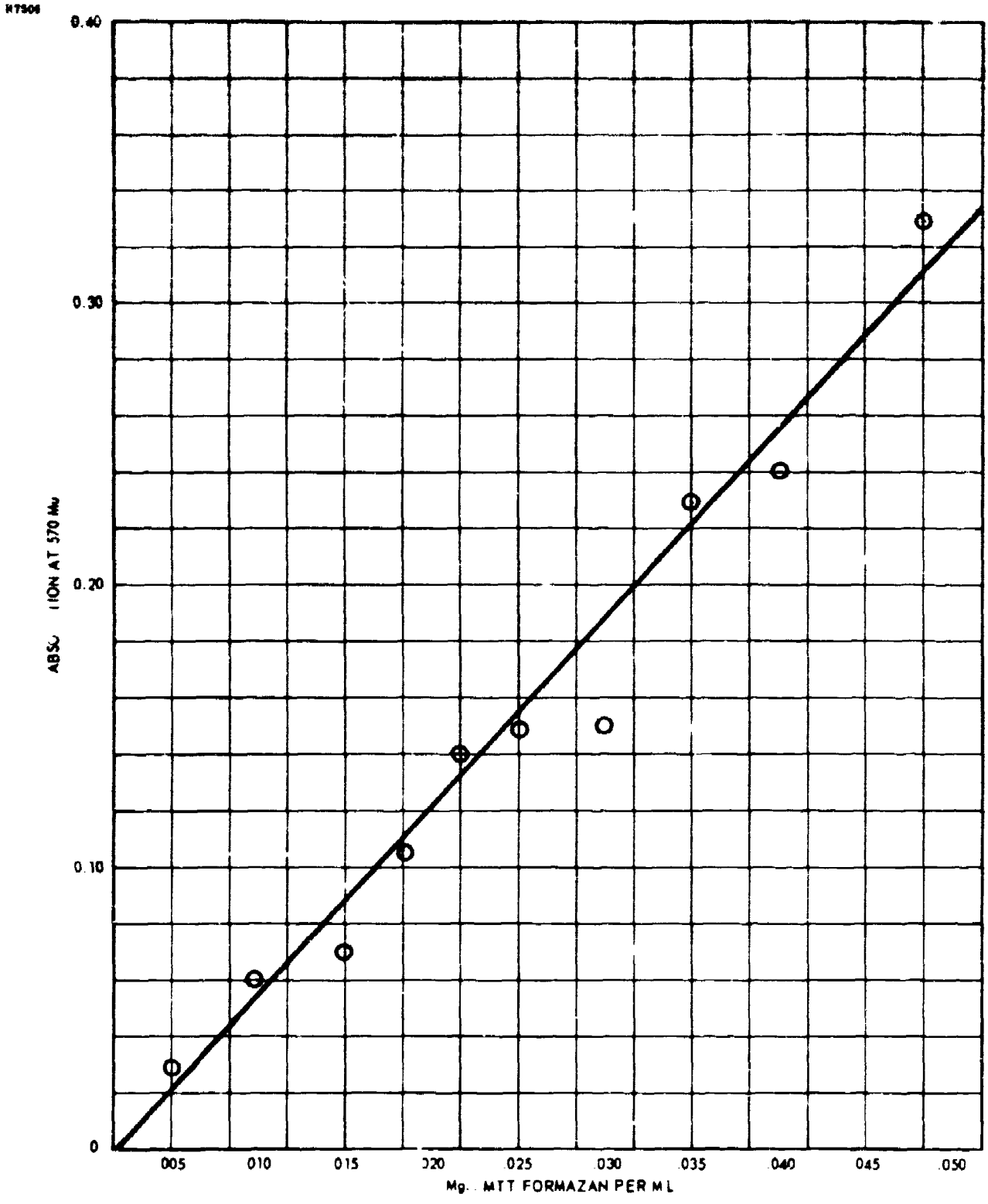

Figure 5. Concentration Curve of MTT Formazan in JP-4 Fuel

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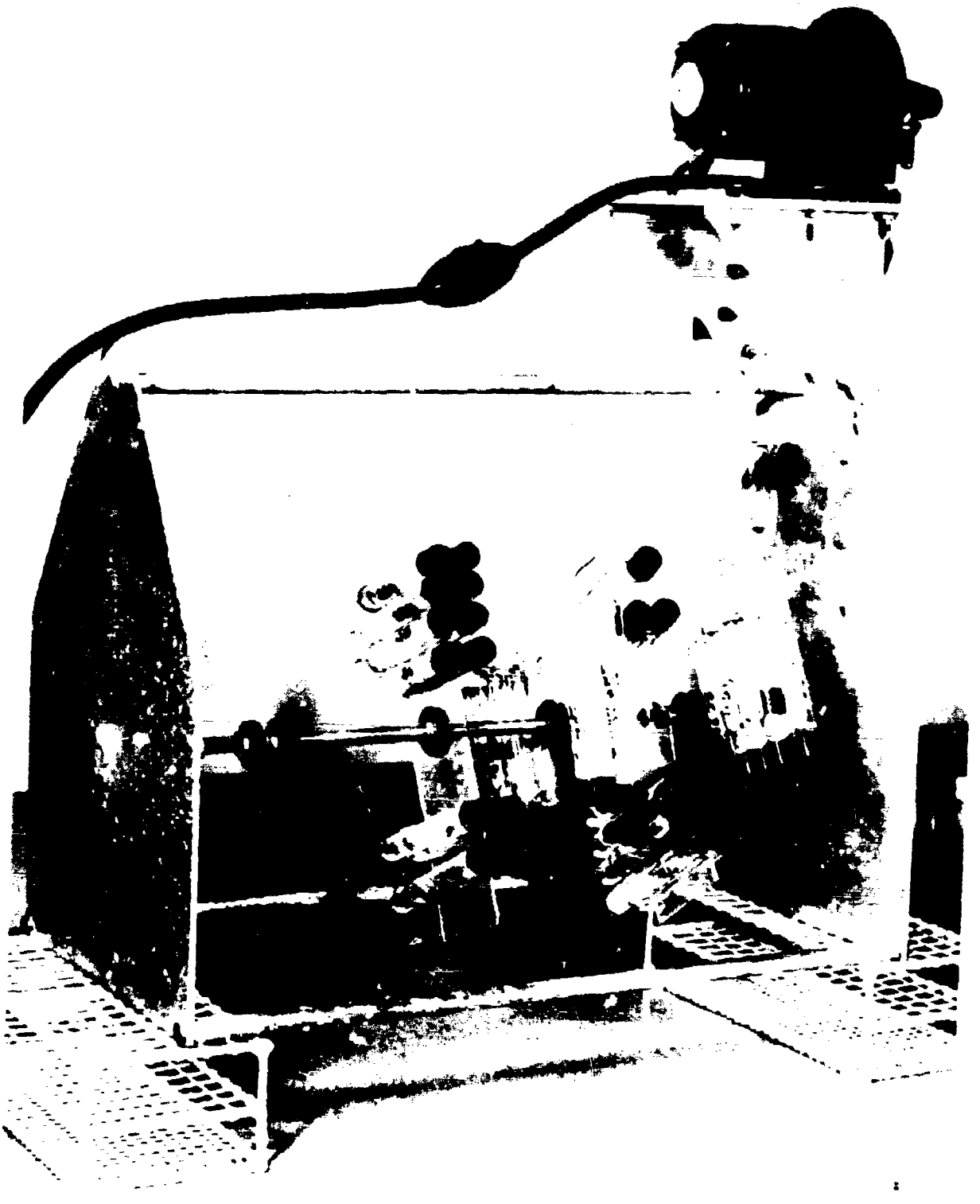

Figure 6. Tube Rotator fo. Tetrazolium Study

TABLE 1

REDUCTION OF MTT BY 20 BACTERIA

Culture	Adsorbancy 570 mμ			
	Time			
	0	3 hrs	24 hrs	48 hrs
87	.000	.032	.07	.128
88	.000	.01	.012	.025
89	.000	.000	.008	.022
90	.000	.000	.002	.010
91	.000	.000	.004	.005
92	.000	.000	.002	.004
93	.000	.000	.01	.015
94	.000	.000	.012	.020
95	.000	.002	.002	.002
96	.000	.000	.02	.035
4	.000	.000	.000	.010
6	.000	.002	.008	.025
8	.000	.004	.002	.005
12	.000	.002	.004	.007
15	.000	.002	.014	.015
16	.000	.002	.002	.005
23	.000	.000	.002	.006
24	.000	.002	.004	.006
25	.000	.000	.004	.006
27	.000	.008	.018	.025
Blank	.000	.000	.000	.000

etc., DPN competes with the dye for available electrons, rather than acting as a transport mechanism to transfer electrons from the enzyme to the dye.

A third series of tubes were prepared in the same manner. To these was added 1.5 ml of $K_3Fe(CN)_6$. 10 mg per ml. After 48 hrs there was no reduction of the dye.

Before testing the reduction of NTV and MTT, the rates of extraction of their formazans from water by JP-4 was compared with that of MTT and tetrazolium blue diformazan. In all cases, 1.0 ml of the formazan, 0.5 mg per ml was extracted into 4.0 ml of sterile JP-4. The results are shown in Table 2. NTV formazan was the most easily extracted, and MTT monoformazan was the second most easily extracted into fuel.

Because of the ease with which the NTV formazan was extracted, an experiment was set up to test the ability of the bacteria to reduce the corresponding dye. 0.4 ml of the dye, 0.5 mg per ml was added, to 3.6 ml of the bacterial suspension in Bushnell-Haas medium. The mixture was overlaid with 4.0 ml of sterile JP-4. Each tube was agitated for 30 seconds on the Vortex mixer and allowed to stand five minutes. The results were negative. After 24 hours, there was no reduction of the dye in any of the 20 cultures. Therefore, even though the formazan was the most readily extracted by the fuel, the bacteria would not reduce the dye. Because a competition between the dye and oxygen may exist, the experiment should be repeated in the absence of oxygen. As mentioned previously, the presumed mechanism by which the dyes are reduced is associated with dehydrogenase enzymes. Three cultures, P1, P5, and J7, were assayed for their ability to reduce tetrazolium salts in the presence of various polymerase substrates. The substrates tested

TABLE 2

RATE OF FUEL EXTRACTION OF 5 FORMAZANS

Formazan	Treatment			
	Spontaneous	With Shaking for 30 sec.	With Shaking for 5 min.	With Shaking for 30 min.
TBD (Tetrazolium blue, diformazan)	-	-	-	+
MTT Mono formazan	+	+	+	+
MTT diformazan	-	±	+	+
NTV formazan	++	+	+	+
NTC formazan	±	+	+	+

++ = complete, very rapid
+ = complete
± = partial
- = none

were sodium lactate, glyceraldehyde-3 phosphate and sodium succinate.

A second set of tubes contained DPN, 0.1 mg, in addition to the substrate. The three cultures selected were previously shown to readily reduce MTT, NTV, and NTC. Formazan production was measured spectrophotometrically in the Zeiss PMQ-2. MTT formazan, NTV formazan, and NTC formazan were measured at 570 mμ, 515 mμ, and 540 mμ, respectively. The reaction conditions and results are shown in Table 3.

The reduction of NTC and NTV appear to proceed more slowly than MTT. However, from Figure 4, the maximum absorbancy of similar concentrations at their peak vary by 5 between NTC and MTT. Reduction of MTT in the presence of the three dehydrogenase substrates was significantly higher than in their absence. The reduction of MTT by 87 was decreased with succinate and lactate in the presence of DPN and increased with glyceraldehyde 3-phosphate. With 88, the activity with succinate was the same as the activity with glyceraldehyde 3-phosphate and DPN. Activities with 27 were variable but were greater with than without substrate.

The data indicate that MTT is more desirable than the other dyes tested, and, that in the presence of suitable dehydrogenase substrates is readily reduced.

Thus far, no attempt has been made to quantitate the results. However, the average cell suspensions contained approximately 10^6 cells per ml, therefore, relatively low levels of bacterial growth can be detected in a short time by this method.

During the next period, experiments will be designed which will define the most suitable dye and substrate combination. Also, attempts will be made

TABLE 3

REDUCTION OF MTT, NTC, AND NTV BY THREE ORGANISMS IN THE PRESENCE AND ABSENCE OF DPN AND DEHYDROGENASE SUBSTRATES

Optical Density of Fuel Extraction

CULTURE	MTT			MTT & DPN			NTV			NTV & DPN			NTC			NTC & DPN			No substrate added		NTV NTC MTT
	S	L	G	S	L	G	S	L	G	S	L	G	S	L	G	S	L	G			
87	.16	.08	.20	.10	.06	.24	.04	.02	.04	.000	.02	.02	.02	.06	.04	.06	.04	.04	.06	.001	.002
88	.12	.06	.18	.08	.06	.12	.000	.02	.06	.000	.000	.04	.000	.000	.02	.000	.02	.02	.02	.002	.000
27	.06	.04	.02	.06	.04	.06	.04	.04	.04	.000	.000	.02	.02	.04	.06	.000	.000	.02	.02	.000	.000
Blank	.002	.004	.002	.000	.000	.000	.000	.000	.02	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000

S = Sodium succinate

L = Sodium lactate

G = Glyceraldehyde-3-phosphate

The reaction mixture contained: 3.5 ml of 3 week old bacterial culture in BH fuel medium. 0.4 ml of dye, 0.5 mg/ml; 0.5 ml of substrate, 1 mg/ml, pH 7.0, 0.1 ml DPN, 1 mg/ml where indicated; and 4.0 ml of JP-4 fuel.
The mixture was shaken on the Vortex mixer for 30 seconds and allowed to incubate at 24° for 30 minutes. After incubation the formazan was extracted into the fuel by vortex mixing.

will be made on the bacterial cultures, enabling quantitation of the dye reduction. A standard curve will be set up which will allow the conversion of optical density of the formazan to numbers of bacteria present in the sample.

c. CO_2 Detection

To evaluate further the measurement of CO_2 and H_2 produced by fuel grown organisms, a series of three studies were set up. These were gas generation tubes, shake flasks and a detection train. The use of gas generation tubes was previously described [4]. The problem of fuel absorption by the septa was overcome by the use of Viton* fuel-resistant closures. Using the same experimental condition described earlier [4], the tubes, containing 13 ml BH medium and 1 ml fuel overlay were inoculated in sets of 4 with cultures 88, 89, 90 and 92. These cultures were previously shown to be associated with corrosion.[4] Each tube was fitted with a miniature magnet so that the contents of the tubes could be constantly agitated. After 12 days of incubation no gas was visible in any of the tubes except for a single bubble in the tubes of culture 92. However, growth was noted at the fuel-medium interface.

In the second study series, using shake flasks, water bottom cultures were used. Because of the varied population and previous results on CO_2 evolution using indicator soda lime, these cultures were considered preferable to single cultures. Those used were a Rimey and a kerosene water bottom. The tests were conducted in duplicate. One flask contained 1.0 g/L $NaHCO_3$ and the other lacked $NaHCO_3$. Each 250 ml flask contained 75 mls of Bushnell-Haas medium overlaid with 3 mls JP-4 fuel, and was capped with a Teflon covered rubber stopper. A port was made in the stopper and sealed with a gas tight

* West Co., Phoenixville, Pa. (Viton, T. M.)

Viton closure. Ten mls of the water bottom cultures was used as the inoculum. Incubation was at 30°C on a rotary shaker. Repeated, periodic samplings were made and analyzed by gas chromatographic methods. No CO_2 was detected in any of the samples.

The third series of studies involved use of a reaction train. The train consisted of a carrier gas (CO_2 free oxygen) which was passed through a tube with drierite and soda lime and then into a $Ba(OH)_2$ solution. These steps were employed to insure the absence of CO_2. The gas then passed into a reaction vessel containing 75 mls Bushnell-Haas medium, 10 mls JP-4 fuel and kerosene water bottom inoculum, 10 mls. Any CO_2 produced by microbial metabolism was carried through and precipitated as the carbonate in the second $Ba(OH)_2$ flask following the reaction vessel. The contents were quantitatively transferred to a graduated cylinder, capped with 2 gas ports. Through one, an excess of H_2SO_4 was added. The solution was vigorously shaken and the gas space above the liquid sampled. No CO_2 was detected by gas chromatography. The $Ba(OH)_2$ flask was replaced and the test repeated after a precipitate was again noted. Although previous results showed CO_2 production, no CO_2 was detected in any of the described procedures. This could not readily be explained especially in view of the formation of precipitate. It was then determined that any CO_2 found would be soluble in the aqueous phase and would require heating to drive the CO_2 off. This was complicated by the fac' that heating also drove off lower boiling fractions of fuel which were carried over into the flasks from the reaction vessel. The presence of volatile fuel fractions and steam in a gas sample interferes with

the gas chromatographic analysis. Consideration was given to gravimetric determination of the carbonate formed. However this procedure would also be fouled by fuel carry over. (See figure 7.)

If CO_2 evolution is to be used as a device for the detection of contamination, other methods of detection will be required. Possible areas will include respirometry over extended periods of time or chemical methods other than those described above.

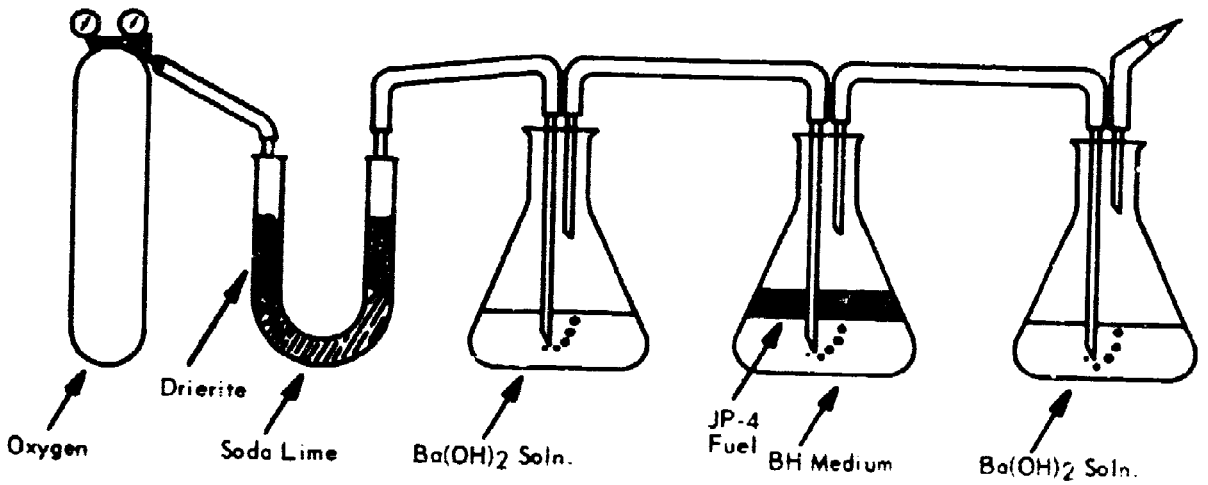

Figure 7. Reaction Train for Detection of CO_2

d. Oxygen Consumption: Polarographic analysis is dependent upon current flow in a solution containing oxidizable or reducible substances. When such a substance is electrolyzed in a cell containing two electrodes, the polarograph automatically records the plot of current versus voltage. As the applied potential is increased from 0, reduction occurs at the cathode, thus causing a rapid rise in current. The rapid rise in current is called a wave, and potential at the midpoint is called the half-wave potential. The half-wave potential is characteristic of the substance being reduced in a given solution. The height of the wave is proportional to concentration of the material in solution and can be used quantitatively [6].

Reduction of oxygen at the cathode in an acid medium occurs in two steps.

(1) $O_2 + 2e + 2H^+ \longrightarrow H_2O_2$

(2) $H_2O_2 + 2e + 2H^+ \longrightarrow 2H_2O$

Formation of hydrogen peroxide occurs at -0.05 V in solutions pH 1-9. Conversion of hydrogen peroxide to water occurs at -0.8 V.

This technique has been used, during this quarter, to determine changes in oxygen concentration in the presence of growing bacterial cultures. A comparison of oxygen concentrations of three cultures was considered. These were (1) a 48 hour culture of 89 in Bushnell-Haas fuel medium, (2) a 48 hour kerosene water bottom culture in Bushnell-Haas fuel medium, and (3) a kerosene water bottom from the original stock culture.

The 48 hour cultures were obtained by transferring 10 mls of culture to 100 mls of BH, plus 10 mls of sterile JP-4 fuel overlay. The cultures

were placed on a New Brunswick shaker at 30°C incubated 48 hours and one ml samples removed for viable plate counts[7].

Oxygen concentration in each culture was determined using an "H-type" electrolysis vessel. One half of the cell contained 8 mls of pH 6.7 buffer plus 1 ml 0.252 M glucose in buffer. The remaining compartment contained the saturated calomel electrode. The oxygen detector consisted of micro-platinum electrode vibrating at a frequency of 7200 cycles per minute and was used in conjunction with the Radiometer polarimeter. Prior to assay, background current was recorded as a function of time over a 10 minute period at a potential of -0.65V versus saturated calomel electrode. One ml of culture was then added to the half cell containing substrate and the decrease in current was recorded at 37°C. A culture of E. coli grown for eight hours in buffered Trypticase Soy plus 0.5% glucose, pH 6.7, was used as control for the system.

Oxygen uptake in the three test cultures was negligible. Uptake did occur with the E. coli culture, however, as indicated by a drop in current from 1.635 μa to 0.07 μa in 35 minutes. The viable count of the test cultures (89 and water bottom on BH) were 100 fold lower than E. coli (Table 4). It is concluded that insufficient numbers of cells were present in test cultures to determine variations in oxygen concentration by this method. Because fuel organisms do not normally get much higher in concentration than observed here, a method to detect this number is critical. Therefore, if oxygen consumption is to be used for detection, other techniques such as manometric methods will have to be considered because the polarographic method is not sensitive enough.

TABLE 4

VIABLE COUNTS OF CULTURES USED IN OXYGEN CONSUMPTION STUDIES.

Culture	Number of Organism
E. coli control	3.4×10^9
	4×10^9
89	2.0×10^7
	1.9×10^7
48 hr kerosene water Bottom	6.0×10^6
	5.1×10^6
Kerosene water bottom Stock-(not transferred)	----*

* No growth was observed on plates of TGY after 4 days indicating that less than 10^3 number/ml were present.

2. Field Methods of Detection

a. Pulse Polarization: The objective of pulse polarization studies is to develop a detection method based on changes in the activation polarization characteristics of an electrode as affected by microorganisms.

The specific objective is to observe the electrode during the very early stages of polarization. The characteristics of double layer capacity and faradic impedance are separated, and the exchange current and Tafel slopes are determined for various degrees of corrosion. The unique X-Y Pulse Polarization method allows one to observe voltage and current of the electrode immediately after a pulse is initiated without disturbing the equilibrium of the system. This information is then correlated with the effect that microorganisms have on an aluminum strip in a suitable media overlayed with jet fuel.

Brief Description of Experimental Technique: The electrical circuit is schematically shown in Figure 8. This circuit is so designed that an aluminum electrode may be subjected to anodic or cathodic polarization in the form of a short D-C pulse. The potential of a reference or non polarized auxiliary electrode vs. the aluminum electrode is monitored on an oscilloscope. After the pulse emerges from the pulse generator it is split into two paths. The two paths are essentially the same, with the cell resistance small compared to R_{LIM}. The voltage across the cell is monitored on the vertical scale of the oscilloscope. Ordinarily the horizontal scale serves as a time representation; however, in this technique the voltage across R_{STD} is additionally imposed on the horizontal amplifier. This causes the position of the pulse on the screen to be displayed by an amount equivalent to this voltage which is directly related to the current across R_{STD}. Thus, the

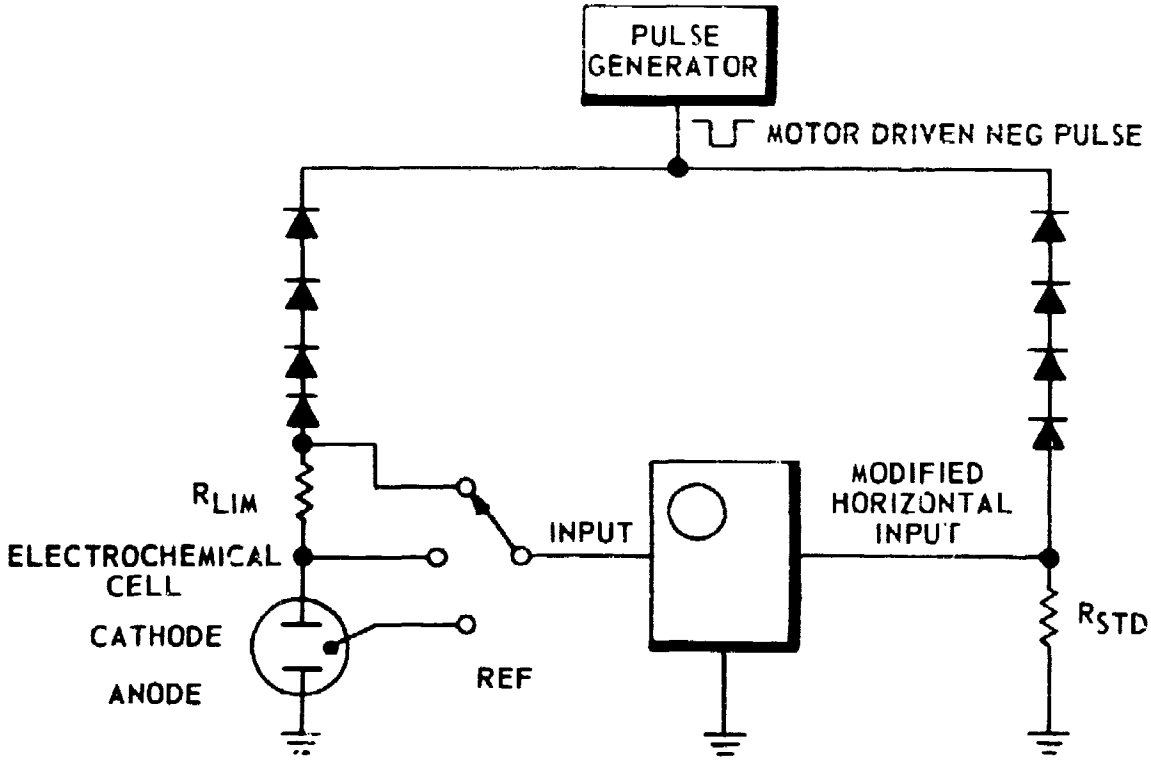

Figure 8. Schematic of Electronic Circuit

[illegible] linearly by a motor driven attenuator and thus a family of voltage-current curves can be obtained for various pulse lengths and at predetermined time intervals after the pulse is applied.

Results and Discussion of the Separation of Electrochemical Characteristics and the Relation to Corrosion Rates: Voltage vs. current tracings for aluminum in certain solutions were constructed using this technique. Initially the following solutions were employed

(1) Sodium sulfate in which aluminum exhibits little or no corrosion.

(2) Sodium chloride in which aluminum exhibits moderate corrosion or rapid corrosion depending on concentration.

(3) Cupric chloride in which aluminum exhibits rapid corrosion.

The corrosion rate was so fast in copper chloride solutions that it was difficult to obtain results of any significant value. Rather than use these solutions it was decided to use various concentrations of NaCl.

The procedure used was to apply a pulse of varying amplitude to the aluminum electrode, and then observe the voltage-current behavior at the electrode and to also observe how the aluminum electrode accepts the potential applied.

The first step was to calibrate the centimeter displacement on the horizontal axis to current. This was accomplished by passing a current across the R_{STD} (100 ohm resistor) (see Figure 2). The voltage E_{LIM} was measured in the vertical direction and thus knowing voltage and resistance the displacement is related directly to current.

[illegible] [illegible] The [illegible] [illegible]

latter is the preferred method, which is accomplished by observing the initial slope of current vs. voltage curves within 0.5 microseconds used after the pulse is initiated. The values varied with the various solutions in the cells used (i.e. 7 ohms for 1 M Na_2SO_4 and 15 ohms for 0.1 NaCl.). The voltage drop due to solution resistance was subtracted from each of the measured potentials. The resultant potential, which is the total overpotential minus ohmic polarization, is here referred to as the overpotential and given the designation η.

It is desirable here to separate the effects of the double layer capacitance from the faradic impedance. Either or both characteristics may be altered by the change in corrosion rates. The double layer charge begins to build as the potential is applied, and within a short time the full charge is established at the interface. This effect may be separated from the faradic effect by measuring the slope of current density-voltage curve as η approaches zero, i.e. as equilibrium is reached. When this value becomes constant, the double layer is established.

For the purpose of this discussion it is desirable to compare the characteristics of an aluminum electrode in 1M Na_2SO_4 and 0.1M NaCl. It was desirable to work in more detail with two particular solutions so that the desired characteristics could be distinctly separated for a more complete analysis. Other concentrations of NaCl and Na_2SO_4 as well as tap water, distilled water, and sodium dichromate were investigated also. The cell was,

[illegible]

[illegible]

platinum reference wire. The aluminum electrode was a small wire with a surface area of 6×10^{-3} cm^2.

In Table 1 the time required to reach the final double layer capacitance is indicated for the two solutions. The following points should be mentioned: It is seen that for the NaCl solution, the double layer capacitance was established within 10 microseconds after the pulse was initiated. However, for the Na_2SO_4 solution, the double layer had not been established even after 200 microseconds. A number of explanations could be offered. First, that the bulky and less electronegative sulfate ion would be slower to arrange at the double layer, or alternatively, the double layer becomes established early but the capacitance is changing due to changes in the surface of the aluminum. An answer to this question may be determined with the aid of a high gain preamplifier for the oscilloscope.

Once the double layer is established the faradic impedance is the limiting effect. In the case of the NaCl solution where the double layer was definitely established the exchange current, i_o, can be calculated from the relationship:

$$i_o = \frac{RT}{nF} \left(\frac{i}{\eta}\right)_{\eta \to o}$$

Where i is the current passing at $\eta \to o$ (equilibrium), R is the gas constant, T the absolute temperature, n the number of electrons involved in the reaction and F the Faraday constant.

Time after Fuse Initiated, [illegible]	[illegible]	[illegible]
-		[illegible]
5	5.35	1.56
10	2.56	1.13
20	1.64	1.13
50	1.22	1.13
100	0.73	1.27
200	0.47	1.36

[illegible]

[illegible] known Tafel equation. [illegible]

$$\eta = a + b \log i$$

where η is the overpotential, and a and b are constants. This equation was first established empirically and later given theoretical meaning. In the plots shown in Figures 9 and 10 the overpotential is sufficiently large so that only the anodic (oxidation) reaction is significant, the opposing reaction is negligible. Linear portions are noted in both plots with slopes of 0.18 for the 0.1 M NaCl systems and a number of slopes for the 1.0 M Na_2SO_4 solution.

Discussion of the Results: Based on the experimental work done in pulse polarization investigations it was observed that there are three electrochemical characteristics of the polarization behavior, any one of which furnish basis for a detection method. These are:

(1) The double layer capacitance

(2) The exchange current

(3) The Tafel slope

Up to the present time consideration has only been given to anodic polarization. Cathodic polarization, which would involve the hydrogen over-voltage reaction, will be included in further investigation. This would double the number of sensitive parameters which might be used in developing a method of to detect the influence of microorganisms on corrosion.

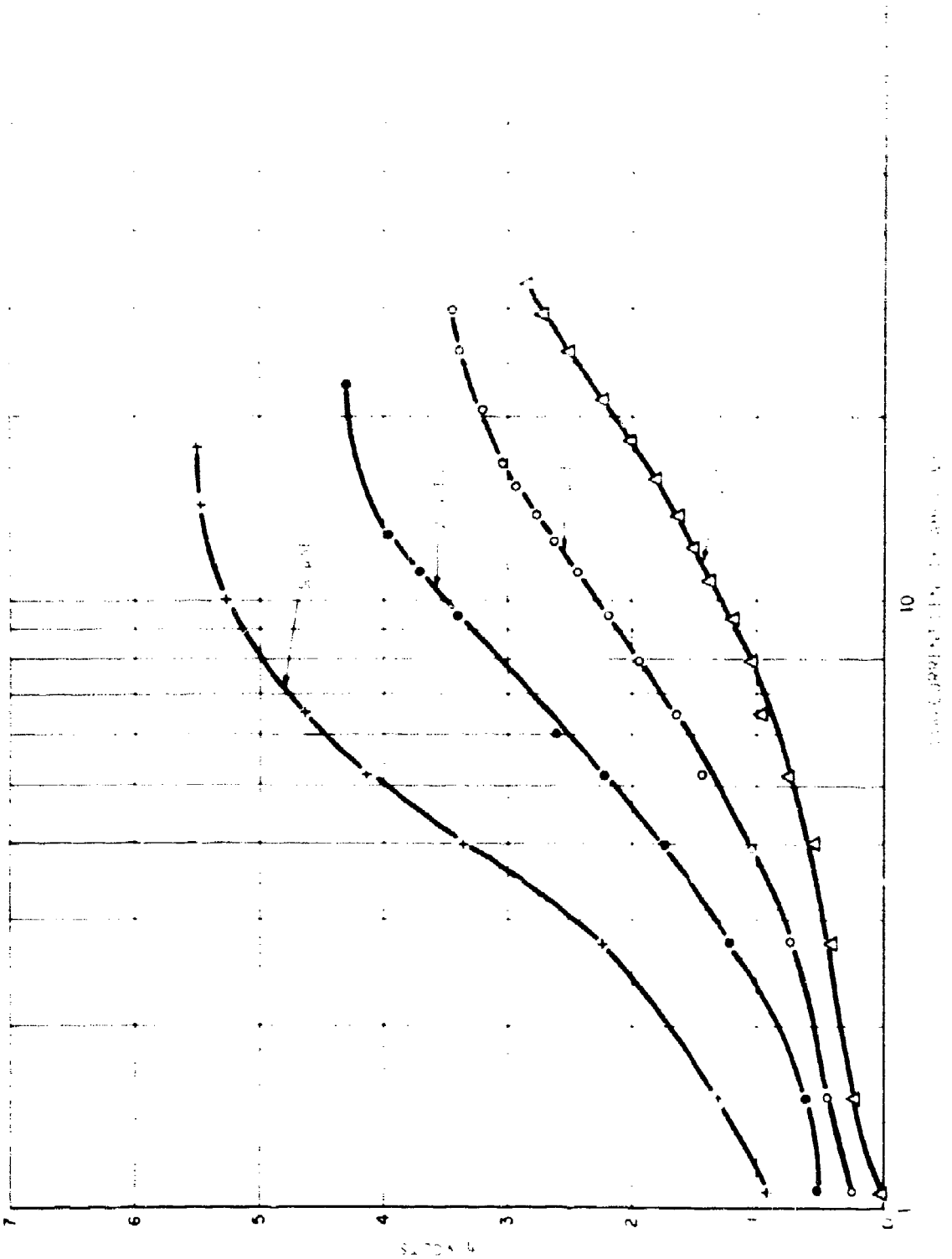

Figure 9. Anodic Pulse Polarization of Aluminum Alloy 7075 in 1.0 Molar Sodium Sulfate

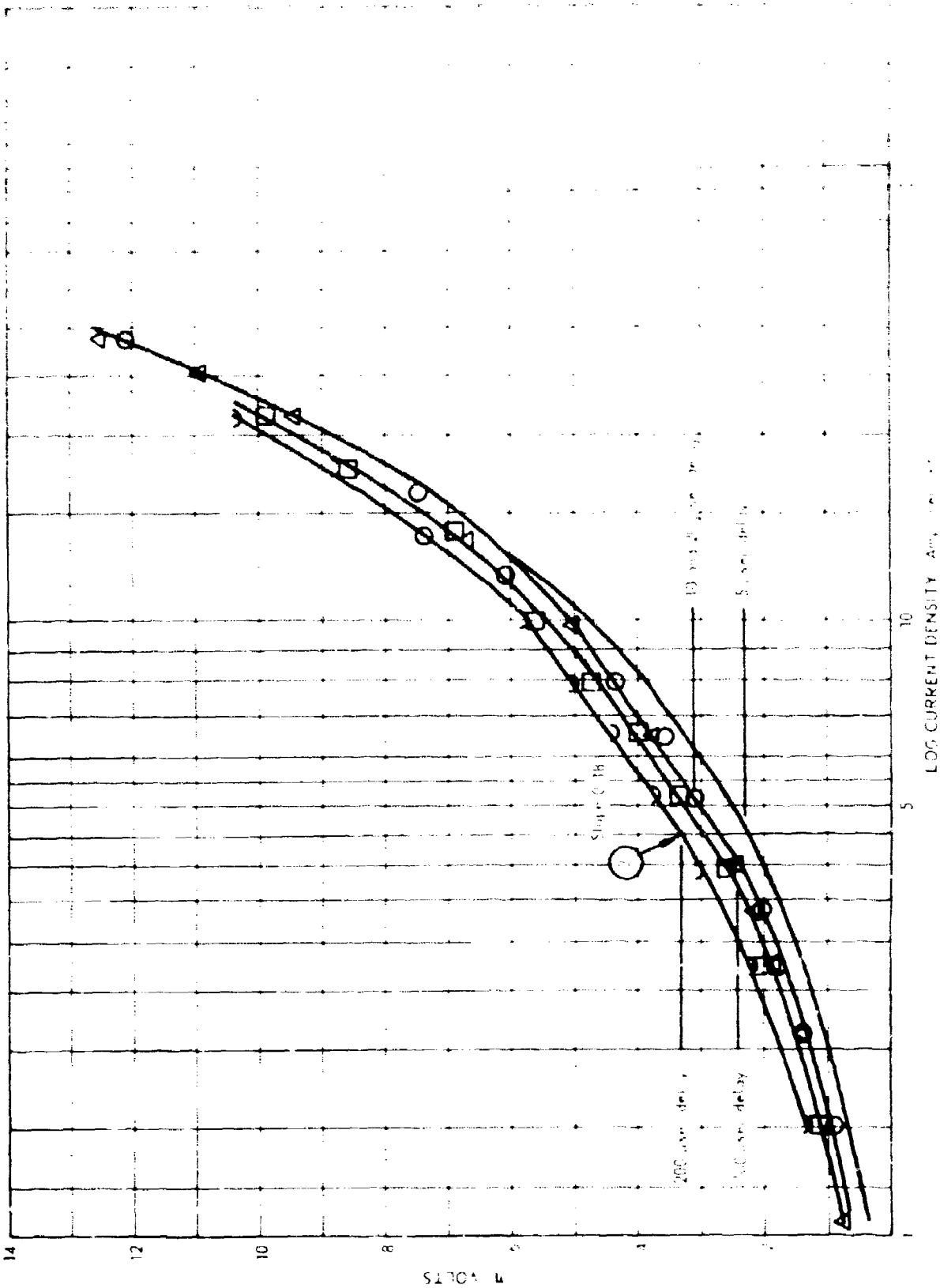

Figure 10. Anodic Pulse Polarization of Aluminum Alloy 7075 in 0.1 Molar Sodium Chloride

[illegible]

[illegible] of the [illegible]. These include changes in surface area, surface configuration, and surface composition. Change in surface area is probably a minor problem. Electrodes with larger surface areas, i.e. [illegible], [illegible] the effect. Change in surface [illegible] [illegible] [illegible] developed on the surface. These are subject to [illegible] current densities. The suggestion has been made that electropolishing or chemically polishing the surfaces might eliminate this [illegible] effect. The third item is [illegible] the greatest cause of concern. [illegible] oxide layer and formation of films on the surface of the aluminum electrode yield non-reproducible voltage scans. A possible remedy would be to start with an extremely clean aluminum surface for each experiment.

Sterilizing the electrodes, electrolytes and cells has offered no problem whatsoever and some preliminary work has been done in Tuckwell-Haas solutions.

The electronic arrangement is satisfactory as it now stands. The current scale has been enlarged by using a Model 533 Tektronix oscilloscope which has 10 times the horizontal magnification as the model 515. It is intended to incorporate a preamplifier with a sensitivity to 1mv/cm in future experimentation.

<u>Plans for the next period:</u> The main objective for the next quarter will be to attempt to relate in a specific manner changes in corrosion to one of the characteristics,

(1) Double layer capacitance

(2) Exchange current

(3) Tafel slope

during anodic and cathodic polarization. An attempt will be made to learn which of the characteristics is most sensitive to the type of corrosion encountered in the systems under study.

In addition to solutions known to corrode aluminum, the media [illegible] to grow microorganisms will be studied and the specific [illegible] separated out. Microorganisms will then be grown in these media in the presence of an aluminum electrode. The electrode characteristics will be observed during various stages of microbial growth. The [illegible] of these microorganisms in the polarization characteristics will be established.

B. Corrosion Studies

1. Screening of Stock Cultures

Long term corrosion studies were begun during the second quarter and three long term studies were accomplished during this quarter. The first study was directed to finding organisms in our stock collection which might be involved in corrosion of fuel cell sludge formation. The second study explored the role of organisms in corrosion and sludge formation. The third study examined the effect of various ions on the corrosion process and the growth of fuel organisms.

An attempt was made to screen organisms for their ability to produce or accelerate corrosion. This study required, ideally, the use of a growth medium, the components of which neither inhibit nor catalyze the corrosion of aluminum alloys.

During the course of this investigation, the Bushnell-Haas medium has been used extensively for the cultivation of fuel organisms, but the effect of this medium on the corrosion process had not been critically evaluated.

A preliminary study of the effect of Bushnell-Haas medium on aluminum showed that corrosion is inhibited by the whole BH (nitrate containing) medium, but is accelerated or catalyzed, by certain di- and tri-valent ions of the medium in the absence of nitrate ion. This observation suggests one of many reasons for the lack of concurrence among investigators regarding the meaning and importance of microbial growth to corrosion in fuel-water-aluminum systems.

In the absence of a medium, other than Bushnell-Haas capable of supporting growth on fuel and simulating the vitamin and carbohydrate impoverished environment of the fuel storage system, the decision was made to attempt to measure the extent of corrosion produced by nitrate-free Bushnell-Haas medium in the presence and absence of bacterial growth.

Screening of 68 stock cultures believed to be involved in the corrosion process was accomplished by growing organisms in the nitrate-free medium in the presence of either alloy 7075 or 2024.

Observations of test and control flasks were made after four weeks of incubation at 30°C. As was expected, 28 out of 72 control strips of the 7075 alloy were corroded, but all 68 strips of this alloy placed in growing cultures were corroded. On alloy 2024 corrosion occurred on 72 of the 88 strips placed in growing cultures, and 6 of the 72 control strips of this alloy were corroded.

Although these experiments did not definitely demonstrate the role of microbes in the corrosion process, two important pieces of information were obtained; (1) on 2024 alloy much more corrosion occurs with organisms than is found in controls, and (2) much more corrosion occurs on 7075 alloy than occurs on 2024 alloy. The latter finding is in disagreement with our previous results, but a similar observation has also been found by Sheldon London at Wright-Patterson, and may be due to the thickness of the aluminum. In our previous experiments the thickness of the 7075 alloy was 50 mil, whereas in the present experiments, 12 mil alloy was used.

2. Metallurgical Examination of Corroded Aluminum Alloys

Because the above results with corrosion of 7075 and 2024 disagree with our previous findings it was decided to determine if the increased susceptibility of the new 7075 was due to thickness, hardness or structure.

A metallurgical examination of certain corroded specimens was made to account for differences in behavior in what was considered to be similar material. Hardness measurements were made on the alloys and in addition cross sections were examined for possible differences in microstructure. At this time the hardness measurements can be reported, the metallographic cross section examination is still in progress. Following are typical values obtained:

Sample	Alloy	Thickness	Hardness*
1	7075	0.050"	153
4	7075	0.012"	66
7	2024	0.012"	110.6
5	7075	0.012"	75.6
3	7075	0.050"	150.5
8	2024	0.020"	116.2
6	7075	0.012"	80.8
2	7075	0.050"	152.9

*Average value of Knoop hardness (150 g. weight)

The 0.050 inch thick aluminum had an average value of 152 as compared with 74 for the 0.012 inch sections. The observation has been made that the 0.012 inch strip corroded at a more rapid rate. This suggests that the thinner strip was subjected to some heat treatment that resulted in precipitated second

phases in a gross manner. This type of precipitate has traditionally yielded alloys more susceptible to corrosion and is particularly noticeable with alloys that contain second phases more cathodic then the matrix. Another correlation is suggested but apparently is not the correct one. This hypothesis would involve the correlation with hardness which in turn is correlated with internal stress known to accelerate corrosion. In such a case the alloy structure with the greater hardness would be expected to corrode at a greater rate. This relationship does not hold in the present instance. It is expected that the microscopic examination of cross sections will allow a better interpretation and correlation of the behavior of these alloys.

3. Growth and Corrosion in Deionized* and Distilled Water

The objective of these experiments was to determine if growth could occur in distilled and deionized water, and to determine if this growth was associated with corrosion. Per cultures from Melpar stocks were used in these studies in addition to four organisms, i.e., Desulfovibrio desulfuricans, Pseudomonas aeruginosa, Cladosporium resinae, Aspergillus niger, received from General Dynamics. The procedure for preparation of control and test flasks was as indicated in the preceding long term studies, except that distilled or deionized water was substituted for the BH-minus nitrate medium. Also, a composite mixed culture composed of five mls from each of the four General Dynamics cultures was included. Test and control cultures were incubated at 30°C on a shake machine.

Corrosion occurred in deionized and distilled water in both the water and vapor phases after only 8 days (Table 6). Although controls also showed corrosion at this time, none were pitted, whereas small pits were detectable in the test flasks.

The 10 corrosion cultures, four General Dynamics cultures and one mixed culture of the four General Dynamics cultures, were again inoculated in distilled and deionized water, overlaid with JP-4, and placed on an incubator shaker. Examination after 24 hours revealed corrosion, as indicated by discoloration of the alloy, had occurred in six of the 10 corrosion cultures in deionized water and seven of the 10 in distilled water. Actual pitting had not occurred in any of the Melpar organisms while distinct pits were visible strips placed in cultures from General Dynamics, i.e., 99, 100, 101, 102 and the mixed culture (Table 7).

*Deionized or distilled water here refers to water treated by passage over an ion-exchange resin or distilled to which organisms suspended in growth media were added.

TABLE 4

LONG TERM CORROSION OF DIESEL IN DEIONIZED AND DISTILLED WATER*

Culture	Distilled Water 7075	Distilled Water 2024	Deionized water 7075	Deionized water 2024
87	+	+	+	-
88	-	-	±	-
89	+	±	±	-
90	-	--	+	-
91	+	-	+	+
92	+	-	+	±
93	-	±	+	
94	-	±	+	-
95	+	-	-	-
96	+	±	±	±
99	+	±	+	±
100	+	-	+	-
101	+	±	+	-
102	+	+	+	+
Mixed	+	+	+	+
Control	C	C	C	C
Control				C
Control	C	C	C	C
Control				C
Control	C	C	C	C
Control			C	C
Control	C	C	C	C
Control			C	C
Control	C	C	C	C
Control	C	C	C	C

*Each test flask contained 100 ml of medium, 10 ml Jet-A fuel and 10 ml of culture

+ = pitting of alloy
- = no corrosion
C = discoloration of alloy strip

TABLE 7

CORROSION OF 7075 AND 2024 ALUMINUM ALLOYS BY CULTURES IN DISTILLED AND DEIONIZED WATER AFTER 24 HOURS INCUBATION.

Cultures	Distilled		Deionized	
	7075	2024	7075	2024
87	+	+	+	-
88	+	+	-	-
89	+	+	-	+
90	+	+	+	+
91	-	-	+	+
92	+	-	-	-
93	+	+	+	-
94	-	+	-	-
95	+	+	+	+
96	+	+	-	-
99	+p	+p	+	+p
100	+p	+p	+p	-
101	+p	+p	+p	+p
102	+p	+p	+p	+p
Mixed	+p	+p	+p	+p
Control	+	+	-	-
Control		+		+
Control	+	+	+	+
Control	+	+	+	+
Control	+	+	+	+
Control	+	+	+	+
Control	+	+	+	+
Control	+	+	+	+
Control	+	+	+	+
Control	+	+	+	+

+ = discoloration of alloy
- = negative
+p = pitting corrosion

To determine if growth could be correlated with nitrite, viable plate counts were made in TGY agar, and potato dextrose agar. Representative Melpar corrosion cultures were also plated. After 96 hours incubation, only cultures [illegible] the mixed culture showed an increase in cell [illegible] between 8 and 96. In culture 99 the bacterial population increased from 2.1 x 10^3 to 1.6 x 10^4 in distilled water, and from 2.6 x 10^2 to [illegible] in deionized water. The population of the mixed culture increased from [illegible] to 1.3 x 10^6 in distilled water, and from 1.1 x 10^5 to 3.4 x 10^6 in deionized water. Cultures 89, 96, 100, and 101 were viable in both deionized and distilled water for 24 hours. No viable cells were present in these cultures after 96 hours. As expected, culture 102, i.e., Desulfovibric desulfuricans, was viable in TGY initially, but no growth was noted after 24 hours. Since oxygen is toxic to this organism further attempts to determine numbers present were conducted anaerobically in Sharpley's agar. Agar dilution tubes of this organism were also prepared by transferring 1 ml of the appropriate dilution to 9 mls of Sharpley's agar. Anaerobic conditions were maintained by use of alkaline pyrogallol and NaOH. The results of this study are not yet available.

TABLE 5

ENUMERATION OF SIX PURE CULTURES AND ONE MIXED CULTURE IN DISTILLED WATER

		CULTURE						
Agar	Time (hrs)	95	96	99	100	101	102	Mixture
TGY	0	4.0×10^{4}	3.5×10^{5}			6.7×10^{4}	3.3×10^{2}	7.7×10^{4}
		1.3×10^{4}	3.7×10^{5}			5.0×10^{4}	4.3×10^{2}	7.4×10^{4}
	24	7.4×10^{5}	3.8×10^{5}			2.3×10^{3}	no growth	1.1×10^{5}
		1.2×10^{6}	4.1×10^{5}			1.4×10^{3}		1.2×10^{5}
	96	no growth	no growth			no growth	no growth	1.2×10^{5}
								1.4×10^{6}
PDA	0			3.1×10^{3}	3.6×10^{2}			1.9×10^{4}
				3.2×10^{3}	3.2×10^{2}			1.5×10^{3}
	24			3.7×10^{2}	1.5×10^{2}			6.8×10^{4}
				2.0×10^{2}	1.2×10^{2}			7.6×10^{4}
	96			1.6×10^{4}	no growth			3.3×10^{5}
				1.6×10^{4}				3.3×10^{5}

*Since cultures 99 and 100 are fungi, these were not plated in TGY
*Cultures 99, 100, 101 and 102 were obtained from Dr. H. G. Hedrick at General Dynamics, Fort Worth Texas

**The mixed culture consisted of cultures 99, 100, 101 and 102.

TABLE 9

ENUMERATION OF SIX PURE CULTURES AND ONE MIXED CULTURE IN DEIONIZED WATER

CULTURE

Agar	Time hrs.	[illegible]	[illegible]	[illegible]	00	[illegible]	[illegible]	Mixture
TGY	0	1.[illegible]x10[illegible] 1.[illegible]x10[illegible]	[illegible].0x10[illegible] 5.[illegible]x10[illegible]			2.[illegible]x10[illegible] [illegible].[illegible]x10[illegible]	[illegible].2x10[illegible] [illegible].[illegible]x10[illegible]	1.1x10[illegible] [illegible]
	24	[illegible].[illegible]x10[illegible] $5.1x10^4$	$1.2x10^5$ $1.0x10^5$			1.[illegible]x10[illegible] 1.[illegible]x10[illegible]	no growth	[illegible] [illegible].[illegible]x10[illegible]
	96	no growth	no growth			no growth	no growth	[illegible].[illegible]x10[illegible] [illegible]x10[illegible]
PDA	0			2.[illegible]x10[illegible] [illegible].2x10[illegible]	$5.4x10^2$ $6.9x10^2$			1.[illegible]x10[illegible] 2.1x10[illegible]
	24			$2.0x10^3$ $1.7x10^2$	$1.5x10^2$ 1.[illegible]x10[illegible]			1.[illegible]x10[illegible] 1.[illegible]x10[illegible]
	96			$1.5x10^4$ $1.6x10^4$	no growth			1.6x10[illegible] 1.[illegible]x10[illegible]

*Table 8

4. Short Term Corrosion Studies

During the third quarter studies included the effect of (1) thin agar films containing viable and nonviable or killed organisms on corrosion, (2) effect of heavy cell concentrations on Mylar tape, and (3) half cell studies. Short term corrosion studies were observed daily for 1 week, with final observations at the end of 4 weeks. Ten corrosion organisms used throughout these studies were chosen on the basis of results observed during the second quarter.

a. Thin Agar Film Studies: When experiments were conducted with aluminum alloy squares and TGY agar, during the second quarter, corrosion occurred most often in plates where the agar layer was quite thin. Studies were conducted, during the third quarter, in which squares of each alloy were covered with thin layers of heavily inoculated agar. Both viable and autoclave killed cultures of 10 corrosion organisms were used in this study.

Thin layers of heavily inoculated agar were prepared on both 7075 and 2024 aluminum squares as follows. Cultures of each organism were obtained by transferring 5 mls of the appropriate culture to 50 mls of TGY broth followed by incubation on a New Brunswick shaker at 30°C. After 48 hours incubation each culture was transferred to 50 mls of warm TGY containing 30 grams of agar per liter. Alcohol flame sterilized squares of each alloy were immersed in the agar-cell solution, drained and placed in sterile glass petri dishes. Since dryness halts the corrosion process, 1 ml of sterile distilled water was added to each dish. In a similar exreriment conducted in BH minus nitrate fuel medium, sterile [illegible] was substituted for distilled water. Test and control plates in both media were incubated at 30°C.

The results show that corrosion, as indicated by deposits on the alloy, occurred on the test and control plates. Controls of 7075 alloy differed from tests in that little or no [illegible] of the metal was observed. [illegible] At the end of 4 weeks no significant difference could be distinguished between control and test alloys.

A similar thin agar film experiment was conducted on [illegible] nitrate fuel agar to determine if differences could be observed in the corrosion by living and dead cells. The cells were killed by autoclaving 15 minutes at 15 lbs. pressure. The suspensions were mixed with agar and the alloys were prepared as before. After 7 days incubation at 30°C corrosion was observed on both test and control squares of the 7075 and 2024 alloys. No significant differences were observed between viable and nonviable organisms because corrosion of the controls was equally prevalent.

To determine if corrosion occurred in BH minus nitrate agar fuel medium, twenty test (each of the 10 cultures in duplicate) and four control plates of 7075 and 2024 alloys were incubated at 30°C and observed daily for 4 weeks.

After two days slight blackening had occurred on many of the test and control alloy squares. After two weeks incubation corrosion was evident on 14 of the 2024 and 17 of the 7075 test squares. No difference in [illegible] test and control squares was noted.

b. Drop Technique on Mylar Tape: Studies utilizing high cell concentrations ($10^7 - 10^9$ cells/ml) were conducted during the second quarter on squares of 7075 and 2024 alloy and [illegible] aluminum foil. Although evidence of corrosion was noted on the alloy squares, [illegible]

was detected. Further study of the drop technique as a rapid corrosion method was conducted in the third quarter with two thicknesses of aluminized Mylar tape* and heavy cell concentrations. Heavy cell concentrations of ten corrosion organisms used in these experiments were obtained by growing the cells 24 hours in flasks containing 100 mls of TGY broth and concentrating the resultant cells by centrifugation. One drop of cells was then placed on ultraviolet sterilized squares of Mylar tape covered with 0.0005 and 0.001 in. thicknesses of aluminum contained in sterile glass petri plates. Sterile TGY broth was used on five control squares of each thickness; both test and control squares were covered with 5 mls of sterile JP-4 fuel and incubated at 30°C. Observations were made daily for two weeks.

Although the aluminum film was removed under the drops of liquid on the thinner aluminized Mylar no differences were observed between control (no bacteria) and the test samples.

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* Obtained from Homar Manufacturing Co., Linden, N. J.

5. Half Cell Studies

Half cell studies were initiated, during the second quarter, to determine the effect of different redox potentials in each side of an H-Cell on 7075 and 2024 alloys. These studies were established in hope that a correlation between rate and pattern of corrosion and differences in potentials produced by growing microorganisms might appear. Although preliminary data indicates that valuable clues to the nature of the corrosion process can be obtained through half cell studies, many difficulties have been encountered with this method during the third quarter. The use of cellophane dialysis sacs to prevent contamination of the uninoculated half cell by bacteria passing through the fritted glass filter dividing the two half cells was unsuccessful since the BH salts and fuel could not be maintained in both half cells at the same level after autoclaving. The imbalance in liquids prevented reliable measurement of potential differences in each of the half cells. Although this difficulty was alleviated by using BH agar as a salt bridge, further problems have been encountered in line voltage current supply to the laboratory area in which these studies are conducted. Although overall results obtained during this period indicate an increase in potential is occurring, day to day readings have been erratic and unreproducible.

Since the BH salts caused deviations in the potentials between the aluminum strips, it was decided to grow the General Dynamics culture of Desulfovibrio desulfuricans 72 hours before inoculation of H-Cells. The cells were washed three times in distilled-deionized water and placed on one side of the H-cells which contained deionized and distilled water with a fuel overlay. The potential difference between the half cells was measured hourly for the first 4 hours and every 24 hours thereafter for 72 hours. The results are shown in

Figure 11. Immediately there was an increase in the negative potential indicating a reduction on the inoculated side of the H-cell. It was then noticed that the potential slowly went from negative to positive. This change in polarity indicates that an oxidation of the aluminum (corrosion), occurred on the inoculated side of the cell.

The control cells, containing no culture; likewise increased in negative potential and then developed a very constant potential both in the deionized and distilled water. Since the initial potential increase occurred in the reaction cell as well as the control, this could be a phenomenon peculiar to this type of cell and not a picture of the overall oxidation reaction. The graph in Figure 11 represents a mean average of (14) H-cells, half with distilled water and the other half containing deionized water. Both sides of all cells contained aluminum alloy bar 2024 connected by a distilled or deionized water-agar bridge. It appears that the rate of corrosion or oxidation is greater in those cells containing distilled water.

Further study of the corrosion process by half cell experimentation is planned for the next month; a culture received from General Dynamics and identified as Desulfovibrio desulfuricans will be used. Pitting corrosion was noted on bars of 7075 and 2024 aluminum alloy placed in distilled and deionized water cultures of this organism after 24 hours. In future studies the effect of changes in potential on the corrosion process caused by high cell concentrations of this organism in distilled water will be investigated.

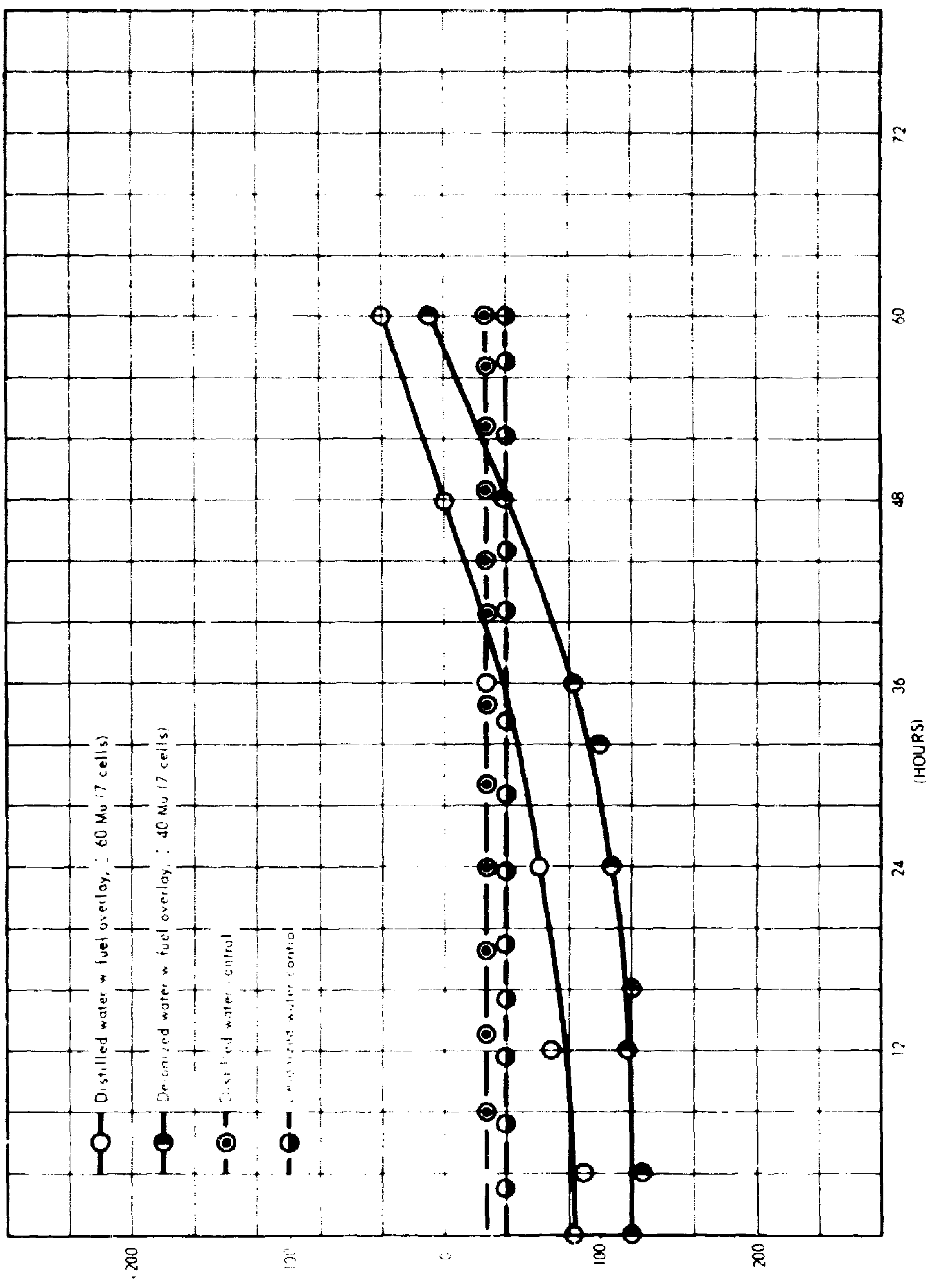


Figure 11. Rate of Corrosion in Fuel Cell

6. Corrosion and Hydrogenase

Studies of the corrosion mechanisms and hydrogenase enzyme activity during the third quarter have centered on five gas producing organisms isolated and assayed quantitatively for hydrogenase activity during the second quarter. Investigation of these organisms, i.e., 88, 89, 90, 92, and 28, during the third quarter, has included duplication of previous results,[4] in addition to determining the effect of nitrate on hydrogenase activity.

Since nitrate ion has been reported as an inhibitor of the hydrogenase enzyme system in the colon-aerogenes bacteria[8], the effect of this ion on hydrogenase activity of fuel isolates was investigated. Three media, i.e., TGY, plus 2 gram of ammonium nitrate per liter, and TGY plus 3 grams of ammonium nitrate per liter were prepared and 100 ml quantities dispensed in 250 ml flasks. The desired culture plus a positive E. coli control culture was then grown anaerobically for 24 hours at 30°C. A five ml inoculum was transferred to identical media, and the new cultures incubated for 24 hours. Cells were then harvested and quantitative determination of hydrogenase activity was determined by the Warburg technique[7]. Results of these assays on three organisms in 3 media are presented in Table 10. Assay of the remaining cultures has been slowed considerably due to mechanical difficulties. These studies are continuing and will be reported in the next quarter.

The results presented in Table 10 show that all three cultures possess hydrogenase enzyme activity when grown in TGY medium, thus indicating that the cultures are capable of utilizing molecular hydrogen. However in the presence of nitrate (2g/liter, twice that employed in the BH medium) a suppression of

enzyme formation is obvious. With three times the concentration of nitrate used in the BH medium, no hydrogenase enzyme was formed. The importance of this phenomenon to the corrosion process, in the light of the nitrate inhibition of corrosion in the absence of microbes, is not known at this time.

TABLE 10

QUANTITATIVE DETERMINATION OF THE EFFECT
OF NITRATE ON HYDROGENASE ACTIVITY

Culture	Ammonium Nitrate grams/100 mls in TGY Growth Medium	uLH_2 utilized/hr.x10^{-8}/cell
E. coli	0	50.2
88	0	57.7
88	0	43.9
88	0.2	6.59
88	0.2	0.
88	0.3	0.
88	0.3	
E. coli	0	42.5
89	0	7.8
89	0	8.4
89	0.2	0
89	0.2	0
89	0.3	0
89	0.3	0
90	0	47.6
90	0	38.1
90	0.2	1.14
90	0.2	2.83
90	0.3	0
90	0.3	0

C. Water Bottom Studies

During the first and second quarter, water bottom studies were initiated to establish (1) a basis for understanding major ecological phenomenon occurring in fuel-water-metal systems, (2) a basis for understanding environmental and biochemical activities of microorganisms as related to corrosion, and (3) clues that can be expanded into methods for preventing deteriorative activity. These investigations were designed around two ecology studies in which major chemical and bacterial population changes in the fuel-water system were followed. The objective of these studies is to determine the time required for oxygen to become depleted and CO_2 to build up during growth in a fuel-water environment in a closed system. This information is needed for an understanding of the metabolism of the organisms for the detection and mechanism studies.

1. Oxygen Concentration

A commercial polarographic oxygen analyzer, manufactured by Beckman Instruments, Inc., has become available recently, for measurement of O_2 concentration of liquids. The sensor unit of this instrument utilizes a silver anode and a gold cathode separated from the sample by a Teflon membrane. The electrolyte is a cellulose-base KCl gel. A built in thermistor insures against temperature sensitivity, allowing measurement over a 15° to 45°C range, with less than ±5% error. The unit can be rapidly recharged thus making reproducible measurements possible.

Oxygen analyses were conducted as follows. A Beckman 777 Oxygen Analyzer was connected to a Leeds and Northrup recorder unit through a small circuit thus allowing expansion of any section of the analyzer scale on the recorder.

Since an adequate flow rate past the oxygen sensor was not obtained in the small system utilized throughout these experiments, it was necessary to run a calibration curve for oxygen in water, correlating the percentage scale on the Beckman Oxygen Analyzer with parts per million by Winkler titration[9]. In a Winkler titration the manganous oxide is acidified in the presence of iodine ion, resulting in liberation of one mole of iodine for each mole of oxygen utilized. The quantity of iodine liberated is determined by titration with standard thiosulfate.

$$O_2 + 2Mn^{++} + 4OH^- \longrightarrow 2MnO_2 \cdot H_2O$$

$$MnO_2 \cdot H_2O + 2I^- + 4H^+ \longrightarrow Mn^{++} + I_2 + 3H_2O$$

$$I_2 + 2S_2O_3 \longrightarrow 2I^- + S_4O_6$$

To correlate the oxygen analyzer scale with concentrations of oxygen three test solutions consisting of tap water, distilled water, and distilled water bubbled with oxygen were analyzed by the Beckman Oxygen Analyzer. Scale readings were then compared to oxygen content as determined by titration with standardized sodium thiosulfate.

Standardization of the sodium thiosulfate was accomplished using potassium dichromate (196mg/25ccH_2O) as a primary standard. Oxygen content was then determined by adding 0.5 ml alkaline solution (40 g NaOH+ 90g

KI/100 ml H_2O) to a known volume of test sample. The solution was acidified with 0.5 mls of 1:1 sulfuric acid and titrated with standard sodium thiosulfate (0.00267N) to a yellow end point. Addition of starch indicator (2g/1000 ml H_2O) at this point resulted in a blue-green color; this solution was again titrated with standardized sodium thiosulfate until the blue-green color disappeared. A blank sample of boiled water served as a control.

In Table 11 a comparison of readings on the analyzer sample volumes and ppm oxygen as determined by the Winkler method is presented. The calibration curve for oxygen content (Figure 12) indicates approximately five scale divisions on the analyzer equals one part per million of dissolved oxygen by weight.

The oxygen sensor unit was inserted in 1500 mls of BH without nitrate solution overlaid with 1500 cc of JP-4 fuel. The water-fuel solution was equilibrated 24 hours at 30°C, approximately 50 mls of water bottom inoculum added, and the system sealed past the sensor with a cork stopper. A magnetic stirrer maintained continuous flow of the sample. By use of the attached recorder the system was constantly monitored during each of the four experiments conducted. (Figure 13).

Run 1 was conducted by placing the oxygen sensor in the fuel layer of the BH without nitrate medium and the flask was plugged with cotton so that the amount of air entering the system was not restricted. In run 2 the sensor was placed in the salts layer of the medium, and the system was closed, but not sealed, thus allowing restricted air to reach the sample.

TABLE 11

OXYGEN CONTENT DETERMINED BY THE BECKMAN OXYGEN ANALYZER VS. OXYGEN CONTENT IN PPM (WINKLER METHOD).

Sample	Volume of Sample Analyzed on Beckman Analyzer	Percent O_2 Beckman Analyzer	Volume of Sample Titrated with Sodium Thiosulfate	PPM O_2
TAP H_2O	100 mls	16.7	9.04 mls	3.3
	200	16.7	18.45	3.2
	300	16.7	27.04	3.1
	400	16.7	36.68	3.1
Distilled Water	200	10.0	13.30	2.3
	400	10.0	26.60	2.3
Distilled Water Bubbled With O_2	100	44	25.0	8.6
	200	41	45.8	7.9

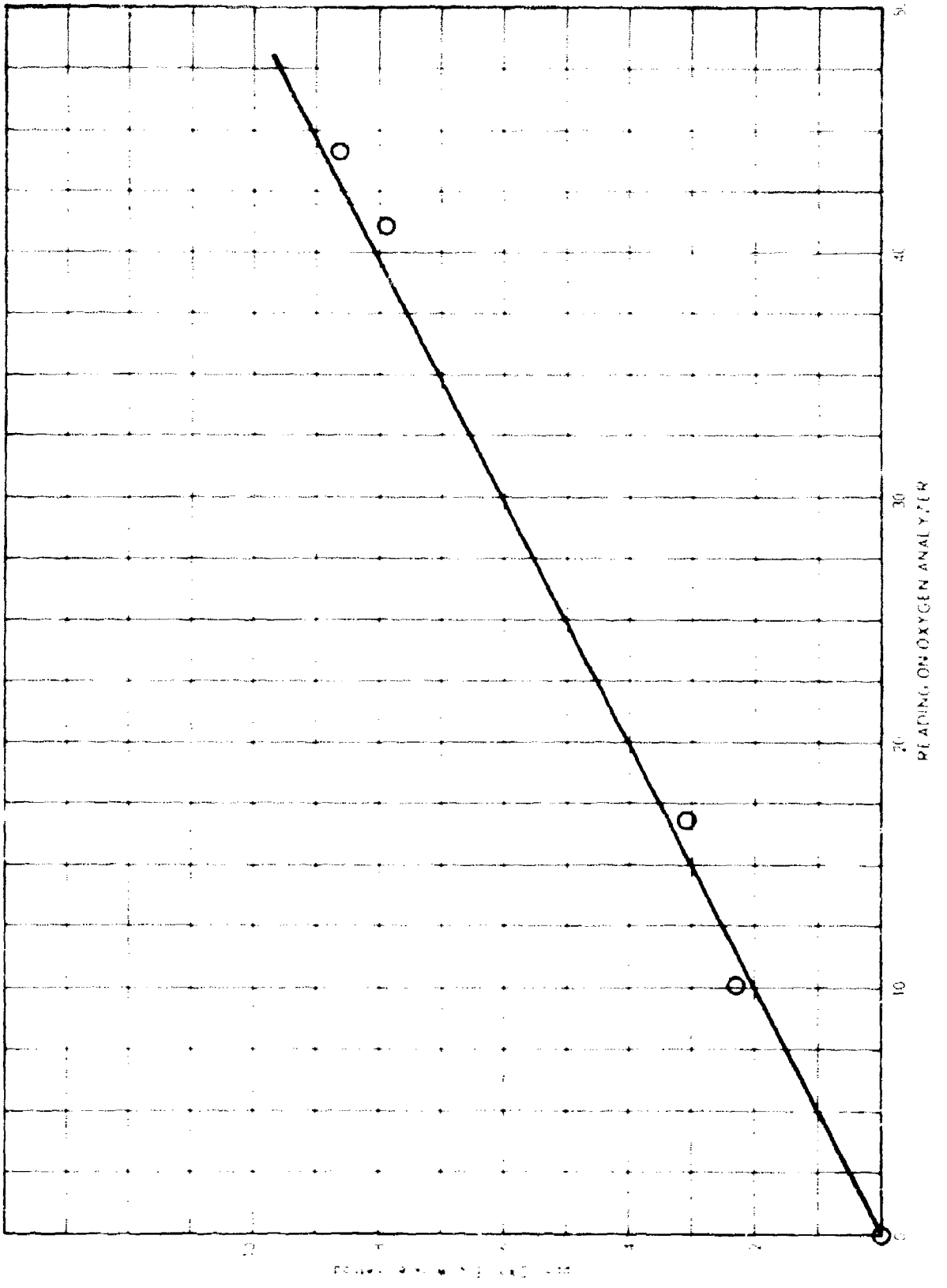


Figure 12. Calibration Curve for Oxygen

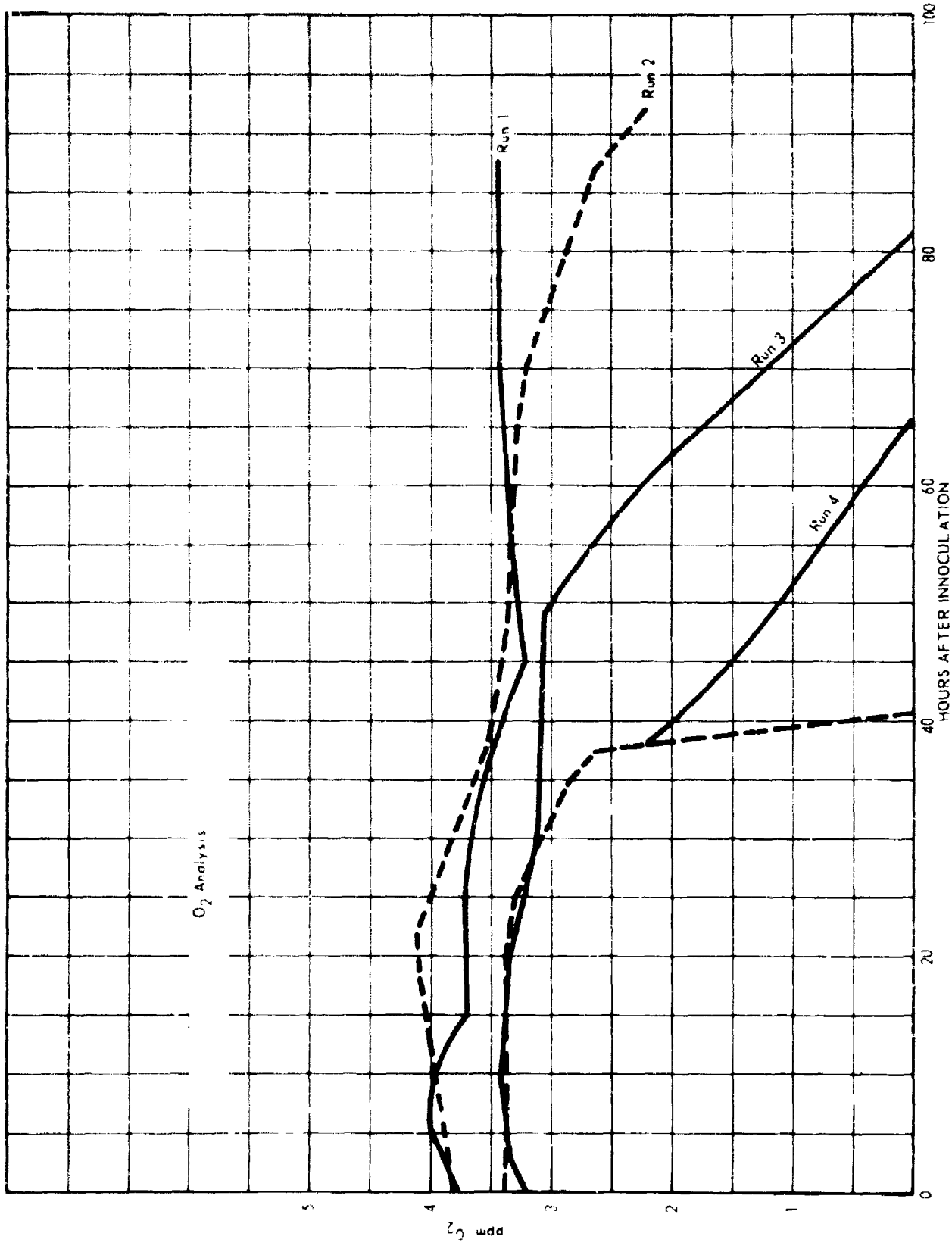


Figure 13. Change in Oxygen Content During Growth of Water Bottom Culture

The third and fourth runs were closed completely, with 25 ml of inoculum for run 3 and 50 ml of inoculum for run 4. The sensor in runs 3 and 4 were in the water layer.

The results in Figure 13 show that no changes in the oxygen concentration of the fuel layer occurred during growth of the water-bottom inoculum (Run 1). Changes in oxygen concentration did occur however in the water layer under partially closed conditions (Run 2). In completely closed systems the rate of oxygen depletion in the medium was a function of the inoculum size. With 50 ml water-bottom inoculum, about half as long (theoretically 46 hours) was required for depletion as was required for a 25 ml inoculum.

2. CO_2 Concentration

The objective of this study was to determine whether the CO_2 concentration of the growth medium in runs 3 and 4 (see Figure 13) increased as the O_2 content decreased. The CO_2 assay concentration was negligible, undetectable by this technique at 0 time, but it increased to approximately 1.3 μ moles CO_2 per ml after 92 hours incubation for run 3, and 48 hours incubation for run 4. This was expected because most of the oxygen had been consumed during growth of the organisms during this time period.

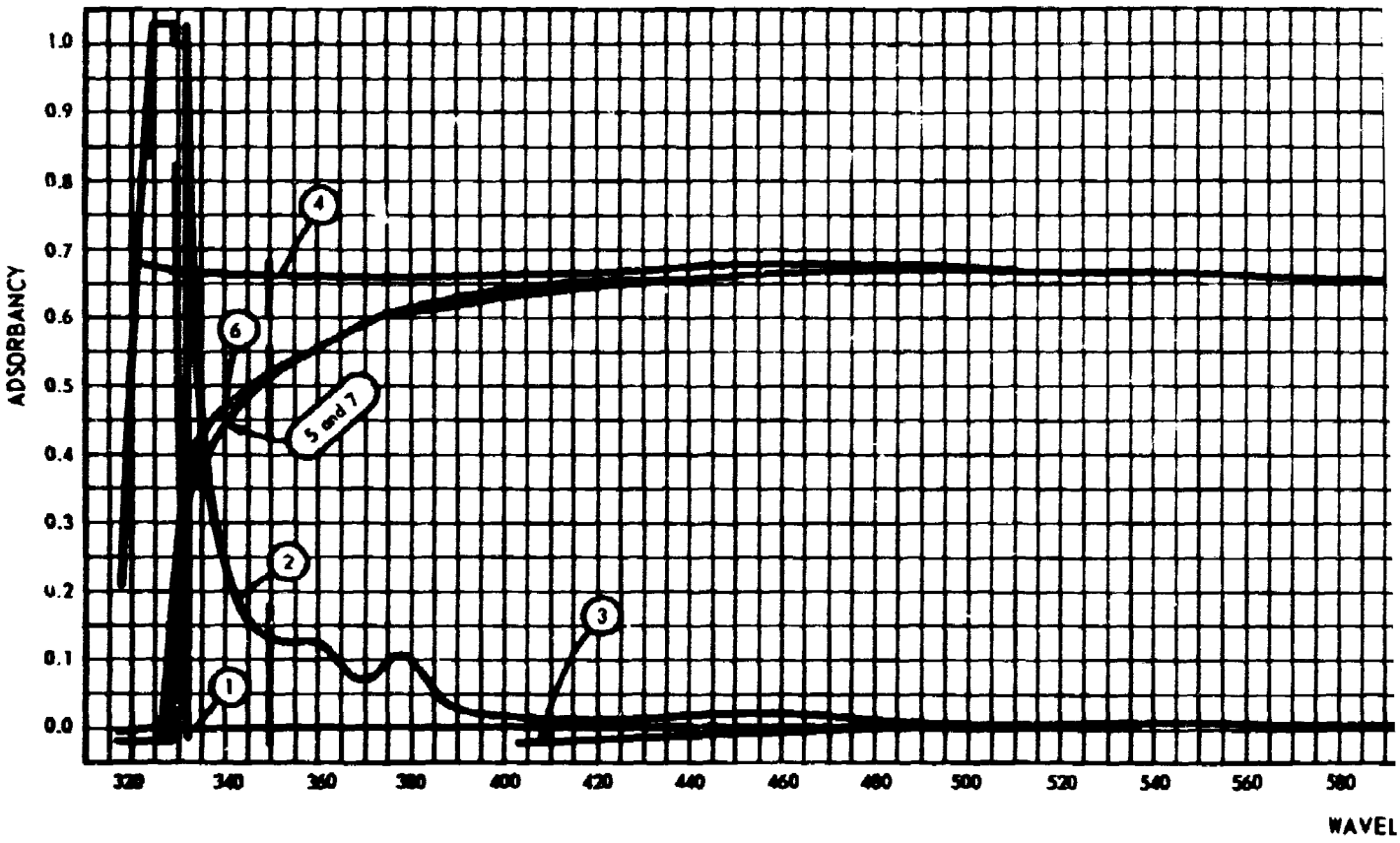
D. Absorption and Fluorescence Measurements

The growth of microbes on fuel is dependent upon the oxidation of hydrocarbons to oxygenated components. Some of the hydrocarbons are oxidized to CO_2, some to cellular components, and some to end products such as slimes and emulsions. The objective for this quarter was to determine if end products could be detected which were related to the corrosion and filter clogging problems. In this study both the fuel and water phases of the growth medium were examined by ultraviolet and infrared absorption spectroscopy and fluorescence measurements before and after growth. The results from these studies are described below.

1. Fuel Soluble Compounds

a. Ultraviolet Absorption Before and After Growth: Microorganisms growing in Bushnell-Haas Fuel medium produce compounds which absorb in the ultraviolet region of the spectrum. The results shown in the First Quarterly Report indicated that microorganisms produced fuel-soluble components in 44 days, which absorb at 310 to 350 mμ. This absorption was though to be due to polynuclear hydrocarbons. During the third quarter, experiments were conducted with fuel which had been incubated with fuel organisms for 5 months.

In some experiments JP-4 fuel was used as an overlay to support growth of the microorganisms in the water bottom (from a kerosene storage tank) while in others JP-4 was used in BH-medium. The ultraviolet absorption spectra observed, using the Cary 14 spectrophotometer, of the fuel before and after growth are shown in Figures 14-17. The same batch of JP-4 fuel was used in


ADSORBANCY
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
320
340
360
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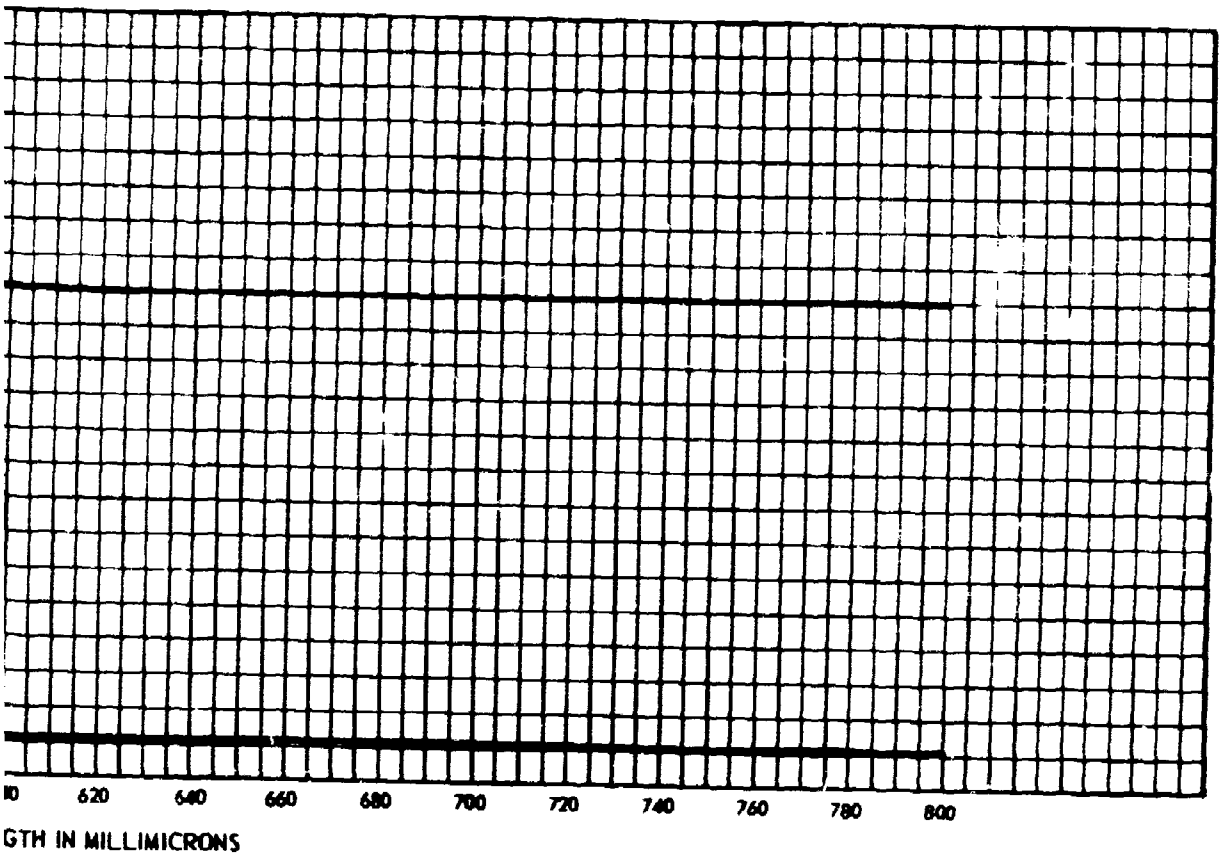

Figure 14. Calibration Patterns of Contaminated and Filter Sterilized JP-4 Fuel

1. and 4. Unsterile fuel vs. unsterile fuel; 2. Overlay fuel from water bottom vs. unsterile fuel; 3. and 6. Overlay fuel from control BH vs. unsterile fuel; 5. Overlay fuel from culture 14 vs. unsterile fuel; 7. Overlay fuel from culture 16 vs. unsterile fuel; Curves 1, 2 and 4 were for unsterilized fuel. Curves 3, 5, 6, and 7 were for millipore filter sterilized fuel.

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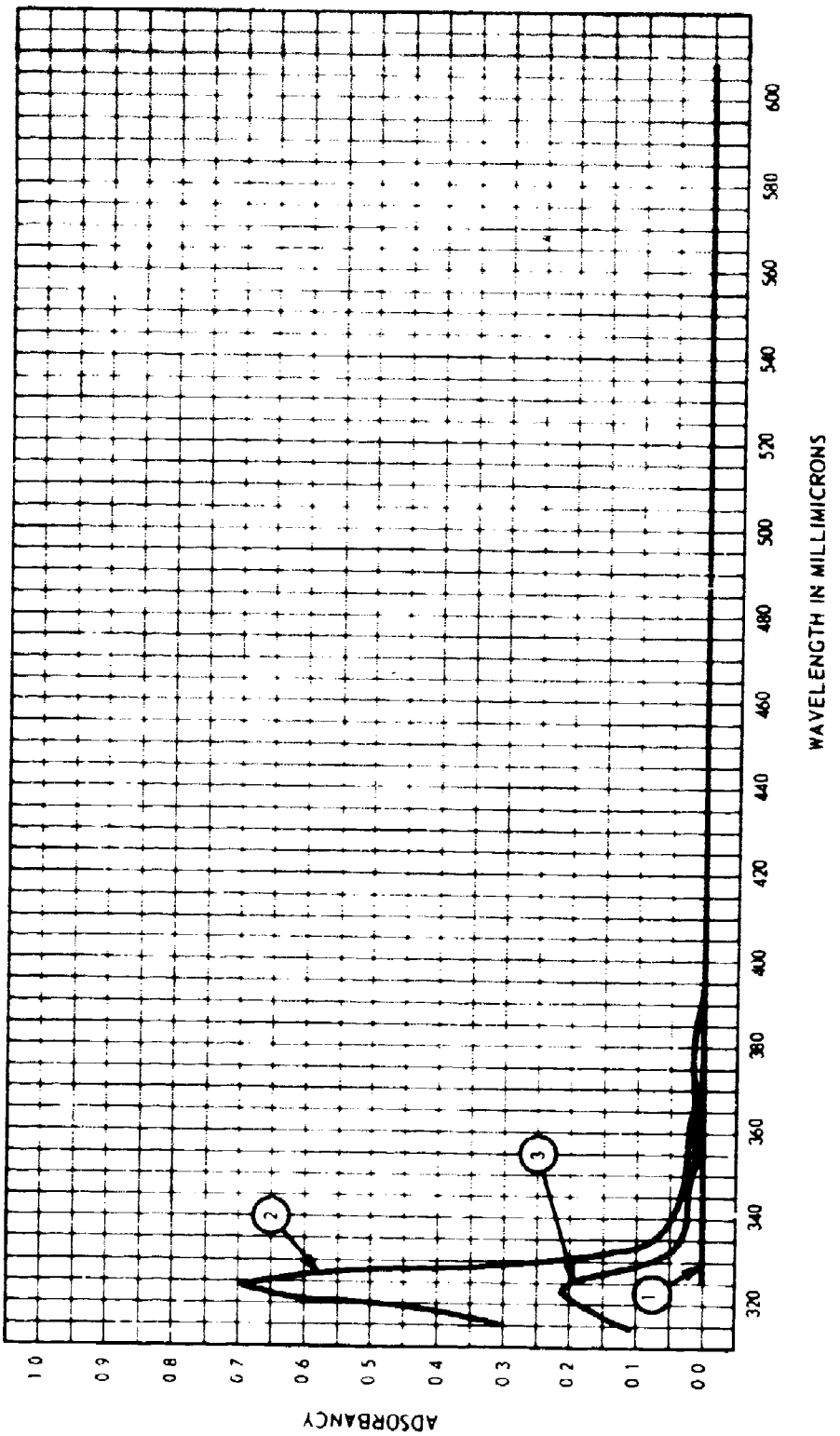

Figure 15. Absorption Pattern of Dilutions of JP-4 Fuel After Growth of Water Bottom Culture

Curves: 1. Unsterile fuel vs. unsterile fuel. 2. 1 10 dilution with unsterile fuel of overlay fuel from water bottom vs. unsterile fuel; 3. 1 100 dilution with unsterile fuel of overlay fuel from water bottom vs. unsterile fuel.

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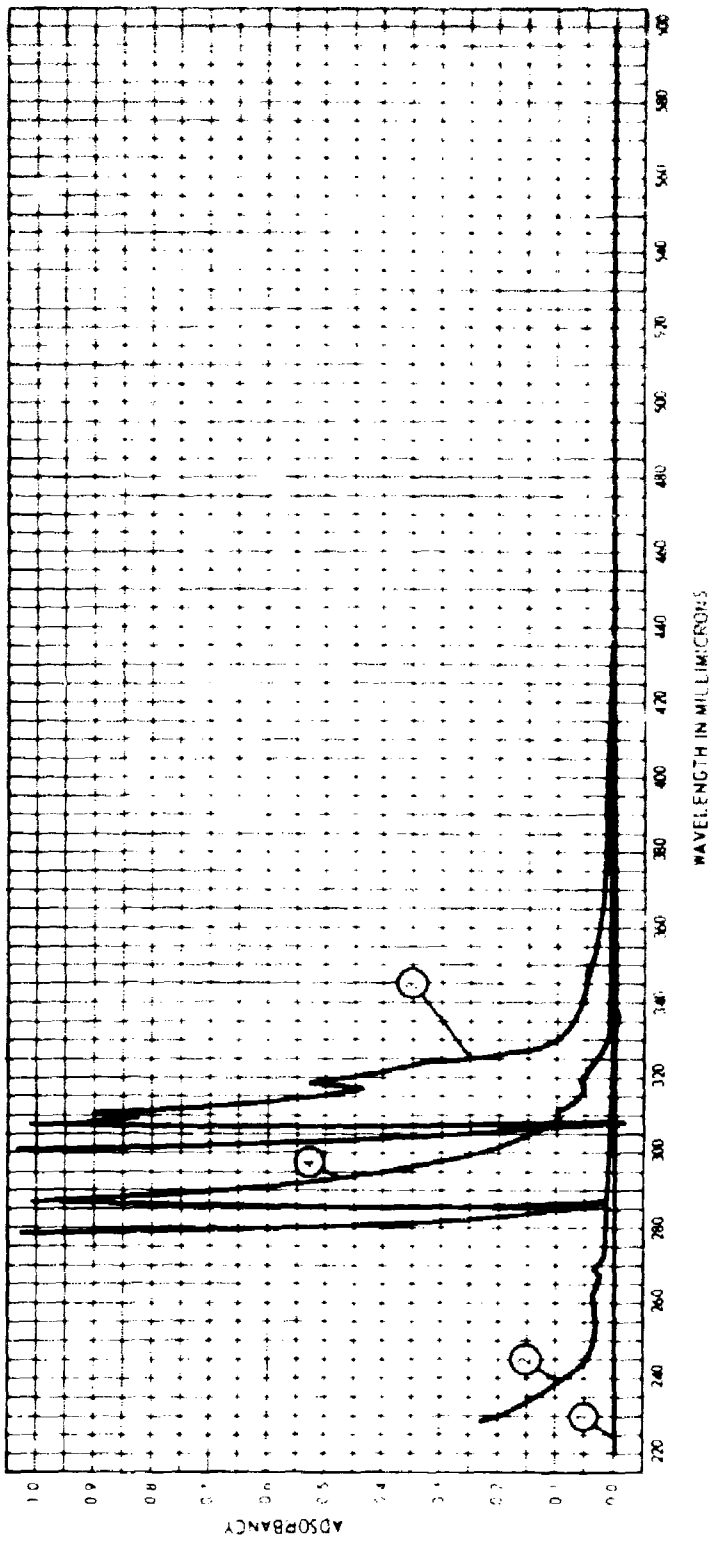

Figure 16. Absorption Patterns of Cyclohexane and Dilutions of Jet Fuel with Water as Reference

Curves: 1. Water vs. water; 2. Cyclohexane (spectroanalyzed) vs. water; 3. 1 10 dilution in cyclohexane of unsterile jet fuel vs. water, 4. 1 100 dilution in cyclohexane of unsterile jet fuel vs. water. Water was used as a reference in these studies.

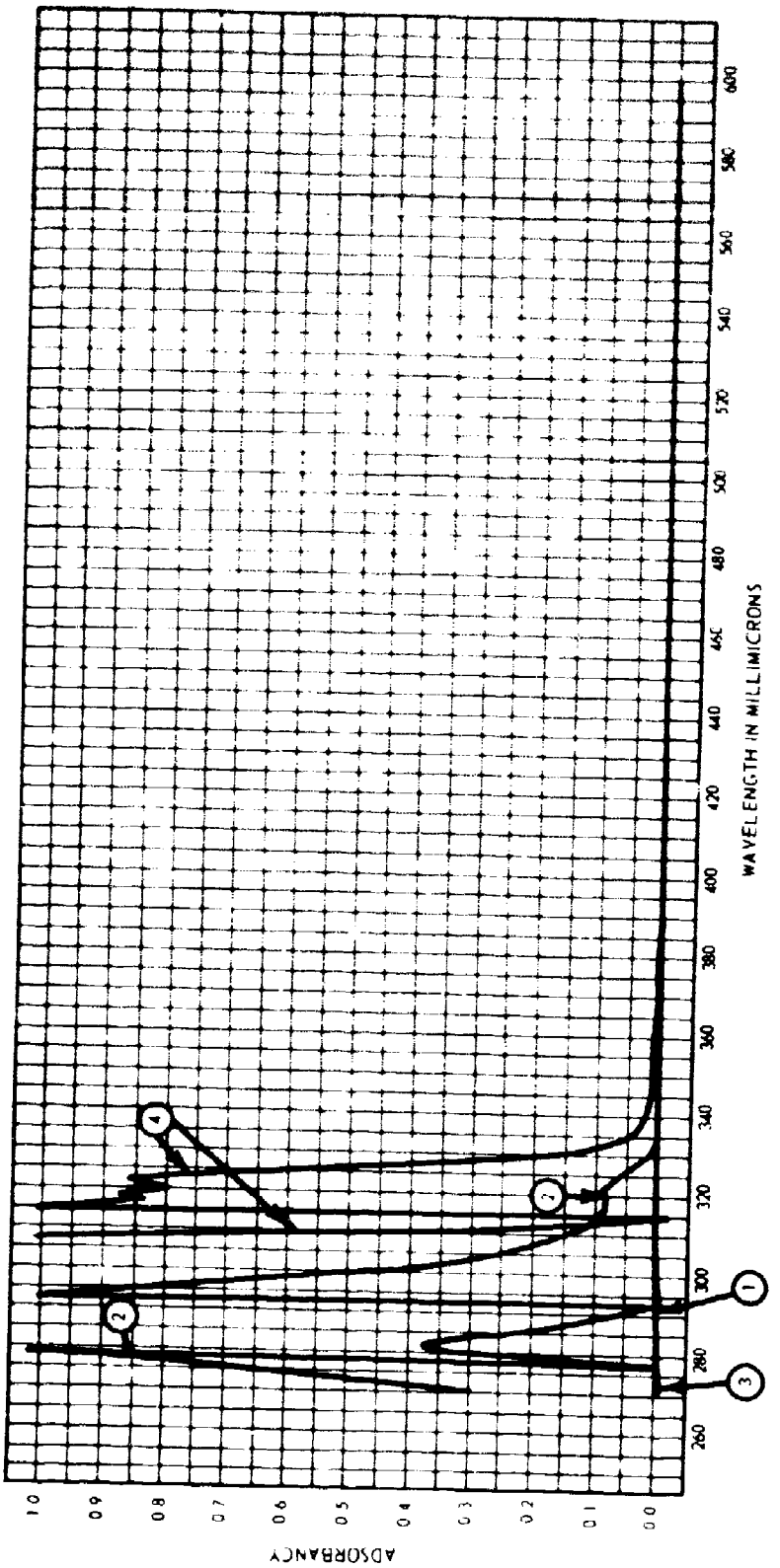

Figure 17. Absorption Patterns of Cyclohexane Dilutions of Fuel Overlay from Water Bottom Culture vs. Jet Fuel Dilution in Cyclohexane

Curves: 1. 1 100 dilution of jet fuel in cyclohexane vs. 1 100 dilution of jet fuel in cyclohexane; 2. 1 100 dilution of water bottom fuel in cyclohexane vs. 1 100 dilution of jet fuel in cyclohexane; 3. 1 10 dilution of jet fuel in cyclohexane vs. 1 10 dilution of jet fuel in cyclohexane; 4. 1 10 dilution of water bottom fuel in cyclohexane vs. 1 10 dilution of jet fuel in cyclohexane. All fuel was from the same batch and was not sterilized.

all cases.

Figure 14: Figure 14 shows the spectral analysis of seven fuel samples. Unsterilized JP-4 fuel was used as a reference in all of these studies, thus only differences in absorption from that occurring in the original fuel are observed. Curves 1 and 4 are base lines for the study which resulted when the absorption of unsterilized fuel was read against itself in both the reference and sample chambers. Curve 2 shows the change in absorption which occurred when the fuel was incubated for 5 months over five gallons of water bottom culture. The peaks (shown) at 360 and 380mμ were not previously shown (First Quarterly Report) because of the dilutions used. When this fuel was diluted (figures 15 and 17) no absorption was observed at these wave lengths. In general, curve 2, Figure 14, is similar to the data previously presented except much greater absorption is shown at 330mμ and below.

Curves 3, 5, 6 and 7 of Figure 14 were fuel samples from bottles containing BH overlaid with millipore sterilized fuel which had been incubated for 5 months. Curves 3 and 6 represent the absorption of uninoculated controls, and curves 5 and 7 represent the absorption after growth of pure cultures of organisms 14 and 16 respectively. The decreased absorption shown in curves 3, 5, 6 and 7 indicates that millipore filtration might be removing some of the components in fuel which absorb between 330 and 440mμ. An experiment to test this hypothesis is shown in paragraph D.1.b. The important observation here is that the water bottom culture produced some fuel soluble components during growth. These compounds were not formed by

the pure cultures.

Figure 15: The curves in Figure 15 show the absorption characteristics of 1/10 and 1/100 dilutions in unsterile jet fuel of the fuel which was over the water bottom inoculum. The base line was prepared as described in Figure 14. The diluted water bottom fuel was compared to the reference fuel. The data is similar to that previously reported except that much greater absorption was apparent at 330mμ and below. About 7-8 times more absorbing material is present in this study than was found previously (O.D. of 0.25 compared to the previous O.D. of 0.03). The reason for the greater absorption is not known, unless it is due to increased metabolic products formed by the longer incubation period; 5 months as compared to 1.5 months.

Figure 16: The curves shown in figure 16 were run to determine the absorption characteristics of jet fuel alone. Spectroscopic grade cyclohexane was used to dilute the fuel, and water was used as the reference. The base line was obtained by using water in both the sample and reference cuvettes. Curve 2 shows that absorption of cyclohexane occurs below 250mμ with no appreciable absorption between 250 and 600mμ. Therefore this solvent should be suitable for diluting the fuel, the absorption characteristics below 320 mμ could be observed. Curves 3 and 4 show absorption of 1/10 and 1/100 dilutions of JP-4 fuel respectively diluted in cyclohexane. Jet fuel at these dilutions has absorption peaks at 318 and 312 and absorbs completely below 290 mμ when compared to water.

Figure 17: The objective of this study was to determine if differences in absorption characteristics could be observed in fuel after microbial growth.

Dilutions of fuel from fuel overlays on water bottom inoculum were compared to appropriate dilutions of reference jet fuel. Cyclohexane was used as diluent in all cases and the base lines resulted from use of diluted reference in both sample and reference cuvettes. The data in curves 2 and 4 indicate that the water bottom culture is producing something that has absorption peaks at 322, 318 and 282mμ.

b. Ultraviolet Absorption of Kerosene and Different JP-4 Fuels: The objective of this study was to determine whether the ultraviolet peaks could be attributed to absorption by kerosene. Also studied were the ultraviolet absorption characteristics of all the JP-4 fuels which have been received.

The kerosene water bottom culture was taken from a storage tank and then allowed to sit for a couple of days before overlaying with JP-4 fuel. The possibility still remained however, that some kerosene component in this water bottom could be responsible for the peaks we observed in Figures 14-17. The absorption spectra shown in Figure 18 show that this possibility truly exists, because kerosene does have absorption peaks at 380 and 360 mμ. It is believed however, that these peaks would have been diluted out more than is indicated; O.D. of 0.1 Figure 14, compared to an O.D. of 0.6, Figure 18, at 380mμ. Therefore the absorption peaks at these wavelengths are still believed to be due to microbial growth.

c. Fluorescence Measurements: The possibility that microorganisms were causing the formation of polynuclear hydrocarbons or conjugated unsaturated compounds was explored by studies examining for fluoresence.

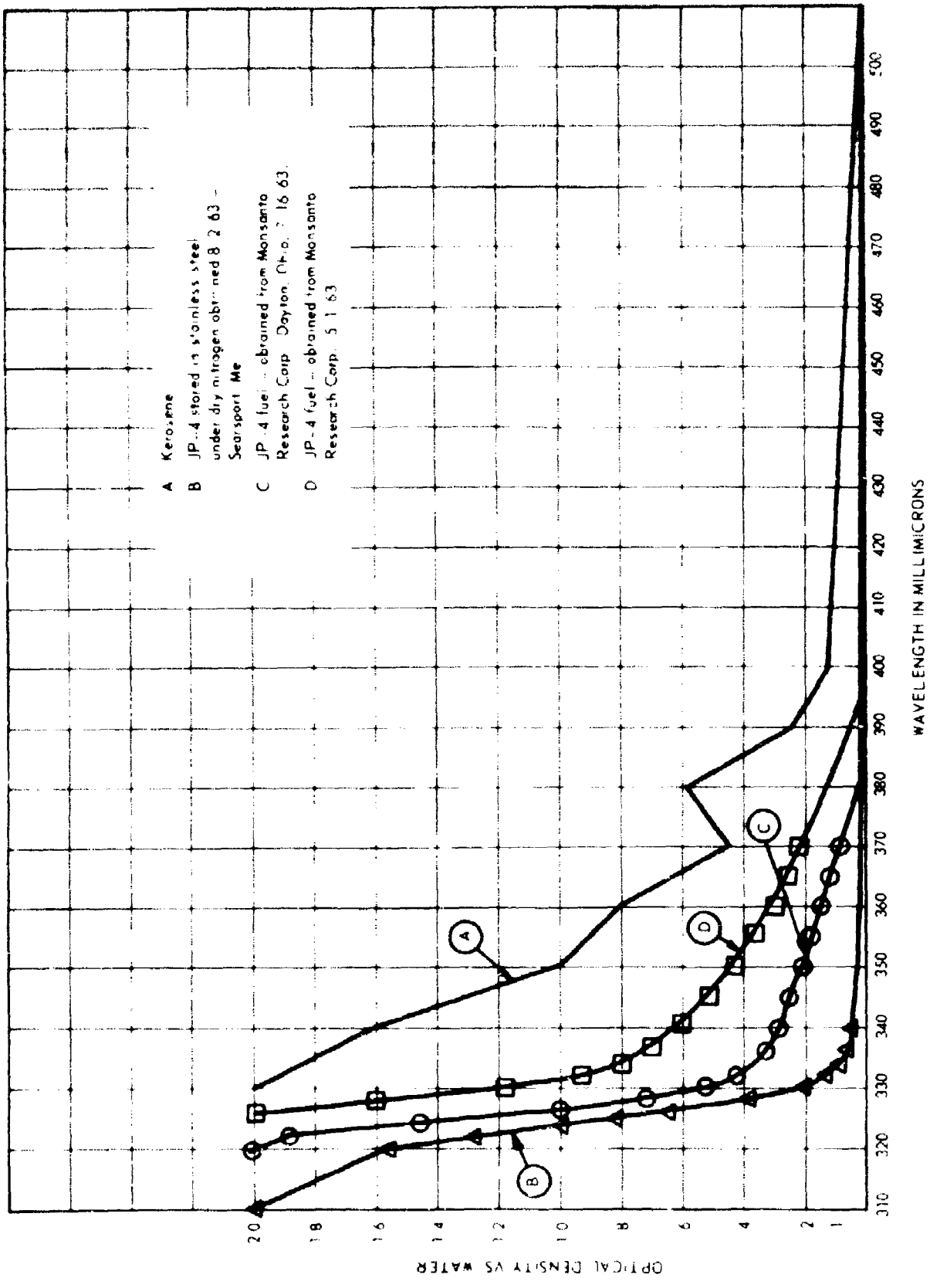

Figure 18. Absorption of Kerosene and Several Fuels vs. Water

The fluorescence of control and water bottom fuels was examined in the Farrand spectrophotofluorimeter. The instrument was calibrated to read 50 units (sensitivity scale X 0.1) with a sample (1 ug/ml) of quinine sulfate dissolved in 0.1N sulfuric acid. In both samples, the control fuel and the fuel overlay, fluorescence was noted at 405mμ with the activation energy wavelength at 355mμ. Fluorescence intensity however was low, about 40 units at the X0.1 scale. Fluorescence at this wavelength implicates polynuclear or conjugated aliphatic hydrocarbons, but because the same fluorescence was observed in the control fuel (no bacteria), their presence had nothing to do with growth.

To make certain that cells were not adding components, the samples were extracted with water solution, and observed for fluorescence at pH 3, 7 and 10. No differences were noted between the control and contaminated fuels.

d. Infrared Absorption of Fuel Before and After Growth: Because the organisms were growing in the water bottom overlaid with jet fuel it was hypothesized that they should be producing oxygenated compounds which would be soluble in the fuel. If this were true, then these compounds might be extracted and characterized by ultraviolet absorption. Preliminary examination of the fuels indicated that some oxygenated compounds might be present. In an attempt to cut down the interferences and to concentrate the potential infrared absorbing compound, both the control and water bottom fuels were fractionated into 31 fractions by distillation and again examined for infrared absorption.

The fuels were fractionated by the following procedure:

500 mls of the fuel were distilled through a 94 cm x 2.5 cm vacuum jacketed column packed with 3/16 I.D. glass helices. The fuel was distilled at 5° intervals, and the reflux ratio of the liquid varied from approximately 20 to 1 at the start of the fractionation to 10:1 at the end of the distillation at atmospheric pressure. The maximum pot temperature at atmospheric pressure was 220°C. Part of the remaining liquid was distilled at a pressure of 10 mm.

It was observed during the distillation that darkening of the fuel in the pot occurred from fractions above 14. The final fraction (No. 30) came off as a colorless liquid, but the liquid in the pot remained as a black dark residue (approximately 75 mls.)

Each of the fractions of the water bottom fuel was examined by infrared spectroscopy. Unfortunately no significant differences could be observed between the control and water bottom fuels and no fuel soluble oxygenated components were formed during growth.

The main conclusions that can be drawn from these studies are: (1) No fuel soluble oxygenated compounds were formed and concentrated in the fuel during growth, (2) the absorption peaks observed at 282, 320, 360 and 380mμ are probably additional polynuclear hydrocarbons or conjugated unsaturated compounds formed by the fuel organisms, and (3) the ultraviolet absorption differences in the fuels before and after growth may be due to contamination by kerosene. No differences in fluorescence, however, were observed between control (JP-4 only) and contaminated (with bacteria and and possibly with kerosene samples).

e. The Effect of Sterilization Techniques on UV Absorption of Fuel: Sterilization of JP-4 fuel is routinely done by Millipore filtration in which filters with pores 0.45 microns in diameter are used. Recently, studies with this filter fuel indicated possible removal of ultraviolet absorbing components from the fuel by sterilization. Accordingly, autoclave, Millipore, and glass frit sterilized fuels have been investigated this quarter.

Three one hundred ml samples of sterile fuel used in the study were prepared by (1) autoclaving at 15 lbs. pressure for 15 minutes, (2) Millipore sterilization, and (3) filtration through ultra-fine fritted glass. A fourth sample of nonsterile fuel was used throughout the study as a reference blank. In initial attempts to compare the four fuel samples, a Bausch and Lomb Recording Spectrophotometer, Model 505 was used. Since the fuel samples absorbed completely below 325 millimicrons, the fuels were diluted with cyclohexane and scanned from wavelengths of 250 to 400 millimicrons. Initial scans indicated a more detailed survey of peaks absorbing between wavelengths of 300-350 millimicrons was necessary.

The spectra of nonsterile fuel diluted 1:2000 is presented in Figure 19, and autoclaved, glass frit and Millipore sterilized fuels are shown in Figure 20. Glass frit and Millipore sterilization appears to yield fuels with essentially the same components, but autoclaved fuel differs from filtered fuels by at least one component as evidenced by the isosbestic points at 273mμ and 257mμ. The importance of this difference could be investigated in growth studies in the future.

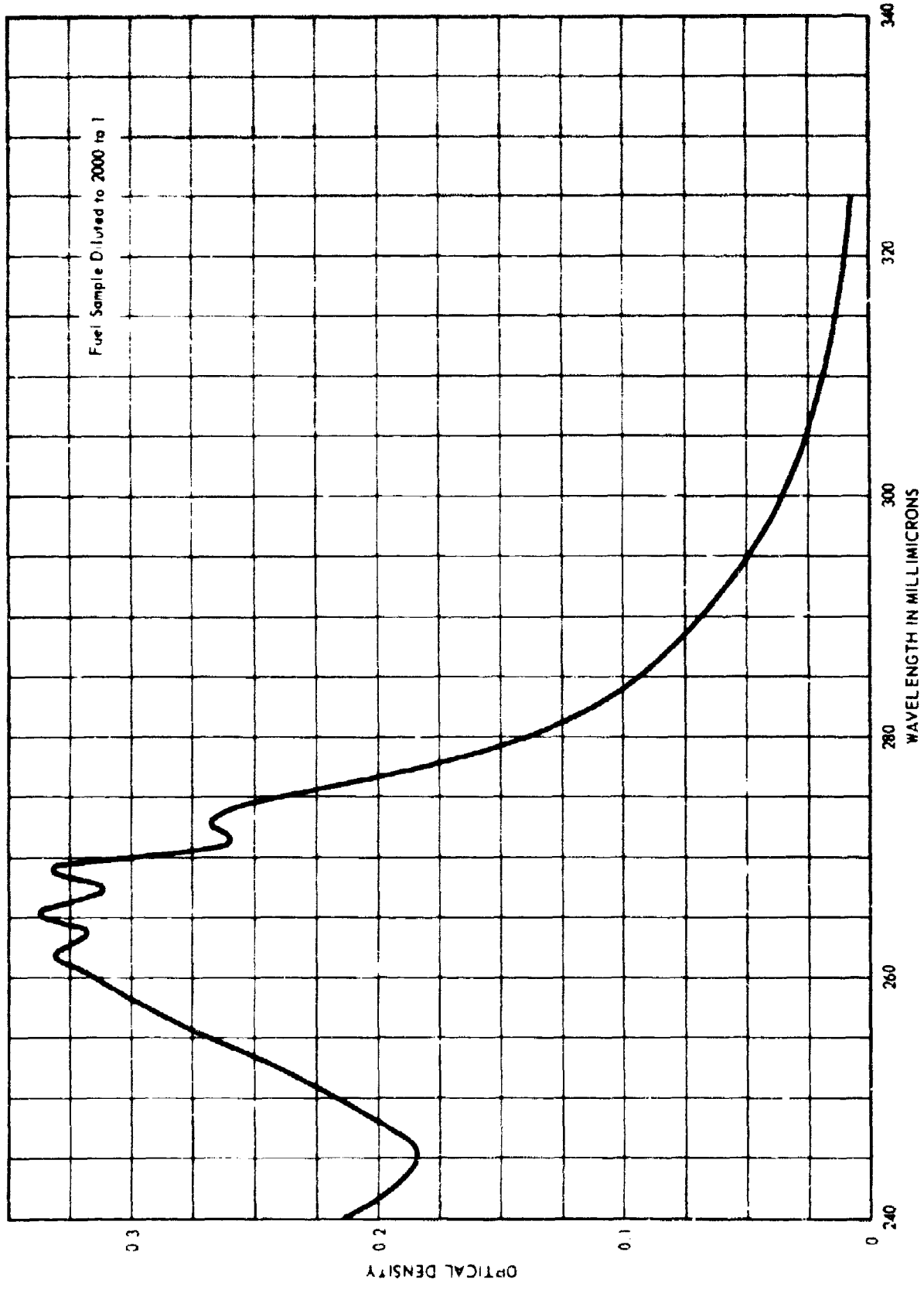

Figure 19. Ultraviolet Absorption of Nonsterile Fuel

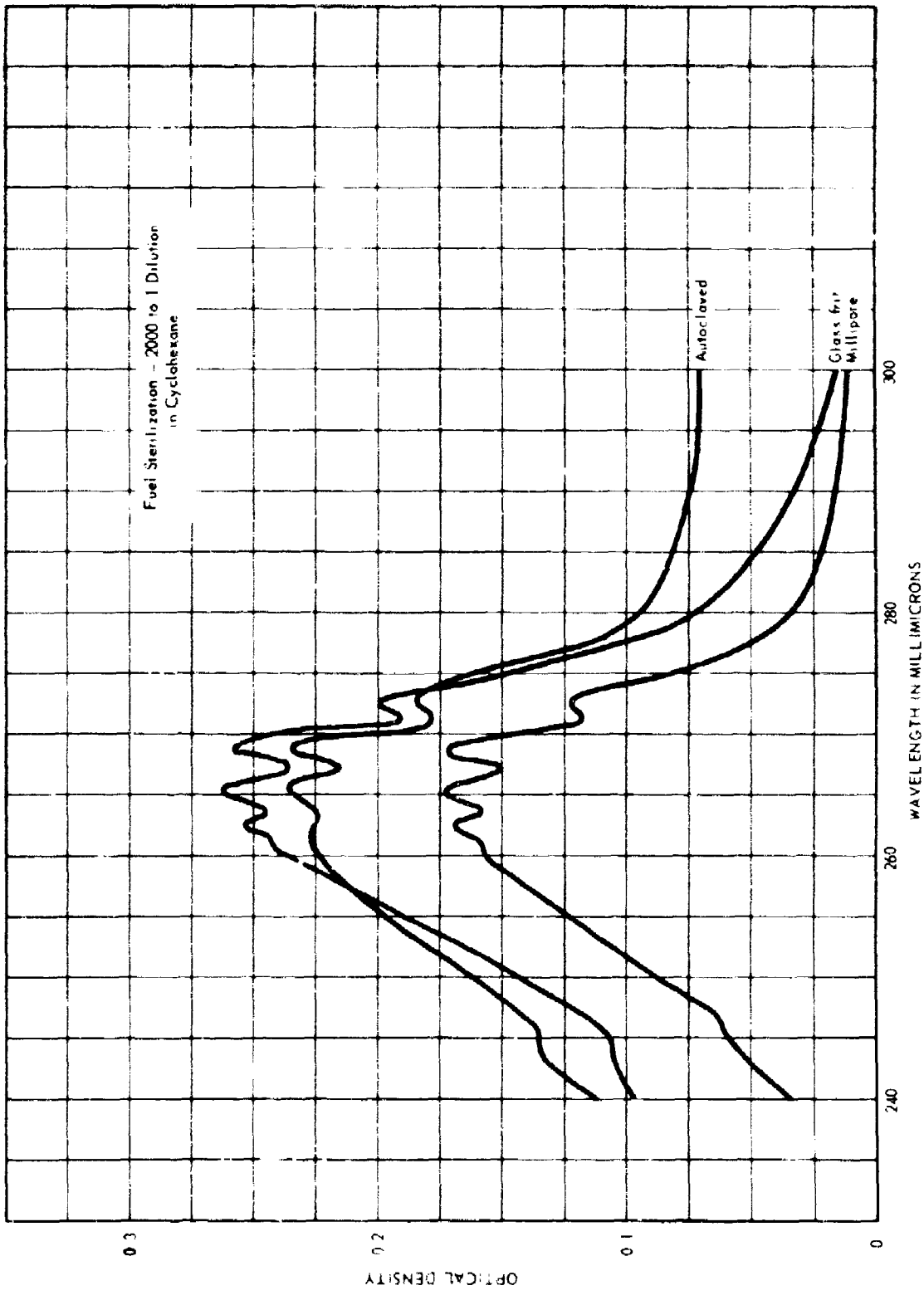

Figure 20. Effect of Sterilization on Ultraviolet Absorption in Fuel

2. Fuel Solubility in Water

a. Microbial Growth on Water Soluble Fuel Components: Preliminary studies showed the extent to which fuel was soluble in water. This was demonstrated by the ultraviolet absorption at 240-340mμ, with a peak at 270 mμ, of water previously overlaid with fuel. The spectra are shown in Figure 21. Experiments were conducted to determine if fuel organisms would grow on these water soluble fuel components.

Initial attempts to determine the effect of microorganisms on water soluble fuel components were conducted by growing three cultures 89, 94, and 96 in BH plus fuel and BH with fuel removed after vigorous shaking. The procedures employed are described in Table 12.

Viable plate counts were made. Table 12 indicated growth occurred in BH medium with fuel and BH medium with fuel removed. Very little or no growth occurred in BH salts only. Changes in absorption at 271 mμ as a measure of growth were complicated by emulsion formation and absorptivity increments did not correspond to increases in cell number as determined by plate count.

b. Use of Water Soluble Fuel Components in Growth: To circumvent this problem of turbidity the water layer was centrifuged prior to ultraviolet analysis the Zeiss spectrophotometer. A mixed kerosene water bottom culture was used as inoculum and the experiment was repeated under the conditions specified in section a. above. The changes in the number of viable cells were not determined.

The results in Figure 21 show the absorption of water soluble fuel components. The absorption of fuel components and emulsions is shown by curves A and B. The absorption shown by curve C is due to the emulsion only. The spectra in Figure 22 show the decreased absorption at 271 mμ resulting from the utilization of fuel as a carbon source by the organisms.

TABLE 12

COMPARISON OF GROWTH OF CULTURES 89, 94 AND 96 ON BUSHNELL-HAAS PLUS FUEL MEDIUM AND BUSHNELL-HAAS WITH FUEL REMOVED AFTER SHAKING.*

Culture	Time	BH-Fuel	BH with Fuel Removed	BH-no Fuel
89	0	4.5×10^5 3.8×10^5	5.1×10^5 4.9×10^5	4.9×10^5 3.7×10^5
	24	8.5×10^6 7.1×10^6	1×10^7 1.3×10^7	8.5×10^5 7.3×10^5
	48	1.1×10^5 9.3×10^4	2.2×10^7 3.4×10^7	6.5×10^5 5×10^5
94	0	1.2×10^6 1×10^6	9.9×10^5 9.0×10^5	9.9×10^5 9.1×10^5
	24	2.1×10^6 1.3×10^5	3.1×10^7 2.7×10^7	2.2×10^5 1.3×10^6
	48	2.5×10^7 2.1×10^7	1.3×10^8 1.2×10^8	1×10^6 2×10^6
96	0	6.4×10^5 6.6×10^5	9.3×10^5 9.9×10^5	3.2×10^4 2.7×10^4
	24	6.3×10^6 6.7×10^6	5.5×10^7 1.8×10^7	1.9×10^5 1.9×10^5
	48	2.0×10^5 2.2×10^5	5.6×10^7 7.0×10^7	1.5×10^5 5.2×10^5

*Experiment was done in duplicate for each of the three cultures. 100 ml of BH was overlaid with 20 ml of JP-4. For fuel removed studies the fuel - BH mixtures was shaken in a separatory funnel and the water layer was removed. Aseptic conditions were employed as previously described. 10 ml of inoculum of each culture, previously grown on BH-fuel for 1 week, was added to the BH salts. 1.0 ml samples were withdrawn at 0, 24 and 48 hours for plating on TGY and 3 ml samples were withdrawn for UV analysis.

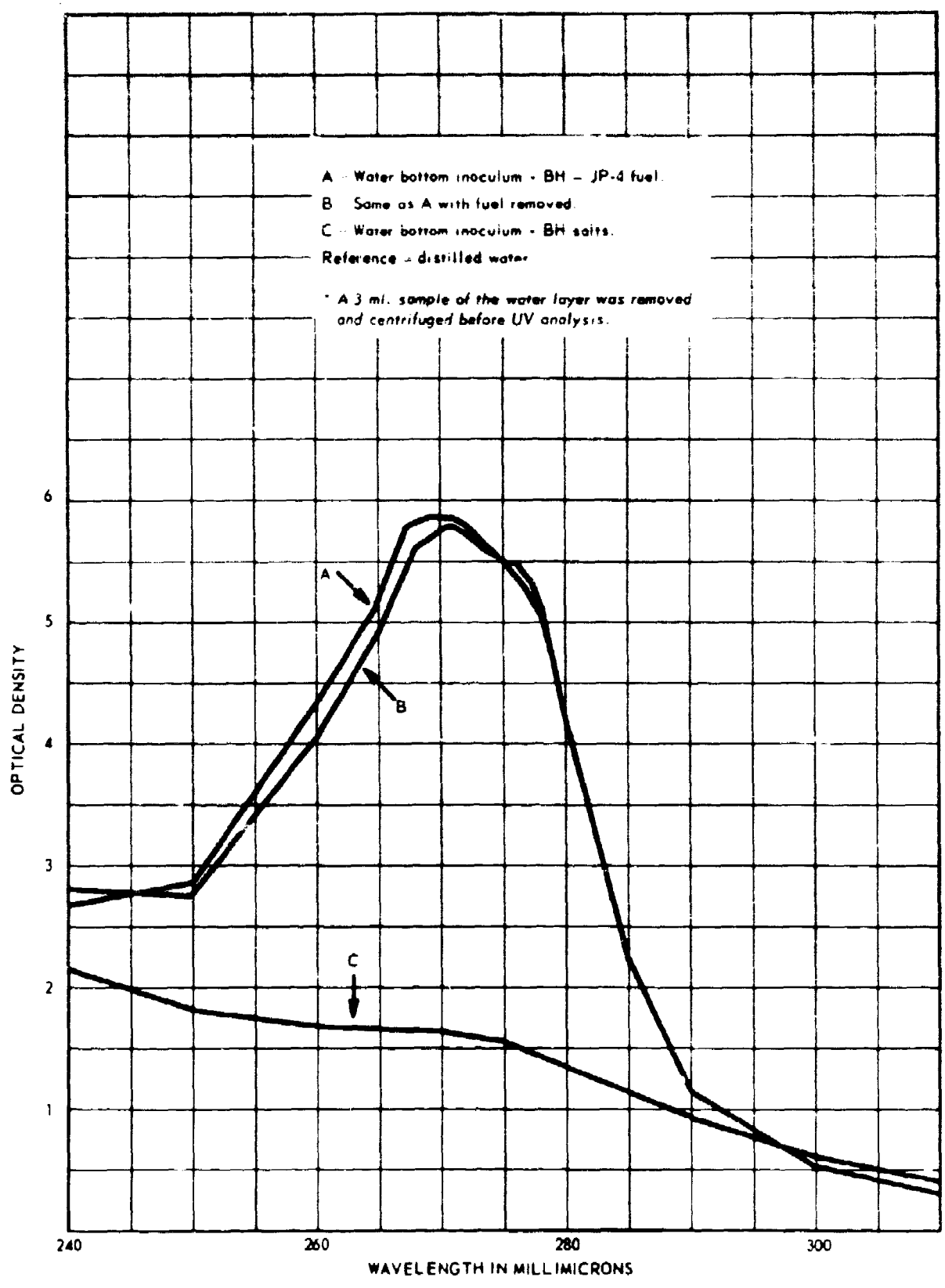

Figure 21. Ultraviolet Absorption of Water Soluble Fuel Components at 0 Time

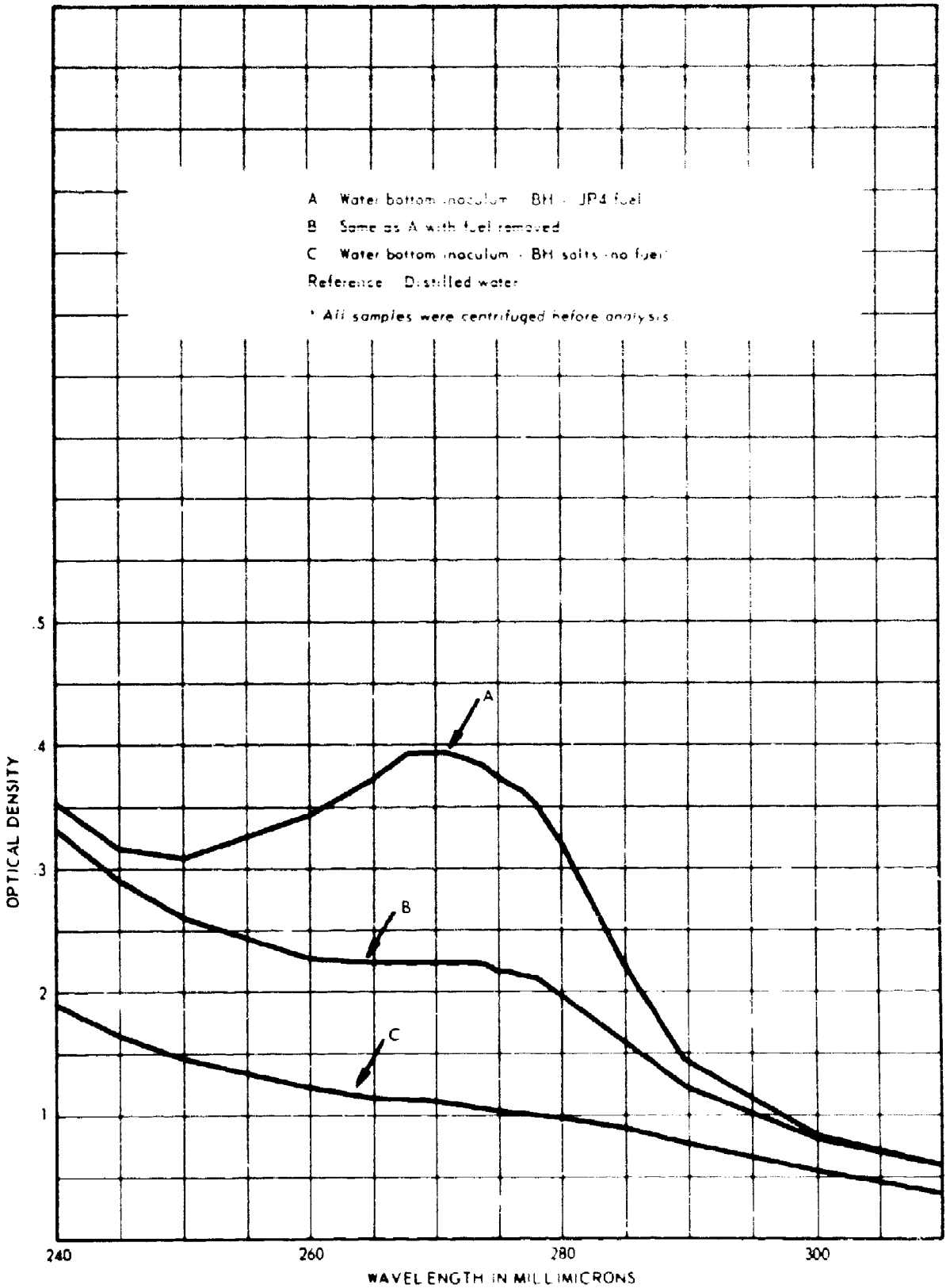

Figure 22. Ultraviolet Absorption of Water Soluble Fuel Components After 75 Hours Incubation with Water Bottom Inoculum

In this preliminary study the time and speed of centrifugation were not controlled and it was obvious that the absorption at 271 mμ could not be compared from one analysis to the next, because different quantities of emulsion were formed in different cultures. However, one can compare curves A, B and C at each of the incubation times. This data is shown in Table 13. The cause of the small difference after 96 hours of growth is not evident. Further experimentation is required to determine the cause of the small difference in absorption observed after 96 hours of growth.

c. Infra Red Absorption of the Water Layer Before and After Growth: Analysis of the fuel layer before and after growth revealed neither great nor significant differences and attention was turned to the analysis of the water layer. Pure culture isolates of cultures 89, 94 and 96 form emulsions in their growth on fuel. Because these organisms were isolated from corroding aircraft fuel tanks and because the current corrosion hypothesis relates the formation of long chain alcohols and acids in low mineral environments with accelerated corrosion, it was decided that analysis of emulsions from these cultures would be desirable. Cultures 89, 94 and 96 were grown for five days in BH minus nitrate on the shake machine at 30°C. Control samples containing no inoculum were treated in the same manner. The aqueous layers were extracted with diethyl ether and dried by evaporation at room temperature in a vacuum desiccator at reduced pressure. This dried material was analyzed by a smear technique and samples from culture 96 were found to contain a carbonyl group as evidenced by the peak at 5.85 μ (Figure 23). The control sample (Figure 24) gave no indication of this material. Samples from cultures 89 and 94 (Figures 25 - 26) do not exhibit strong absorption in this region, but, do indicate the possibility of the presence of a carbonyl group.

TABLE 13

UTILIZATION OF WATER-SOLUBLE FUEL COMPONENTS BY WATER BOTTOM CULTURE

Incubation time (Hours)	Absorption at 271mμ by BH*		Difference
	+ fuel	fuel removed	
0	.586	.580	.008
25	.496	.418	.078
47	.582	.497	.145
71	.474	.310	.164
75	.394	.226	.168
*96	.475	.349	.126
150	.553	.292	.266

*This is absorption after centrifugation. Different centrifugation conditions were employed for samples taken at each time. Theoretically, if identical conditions were employed the absorption of the BH + fuel would be identical for each time.

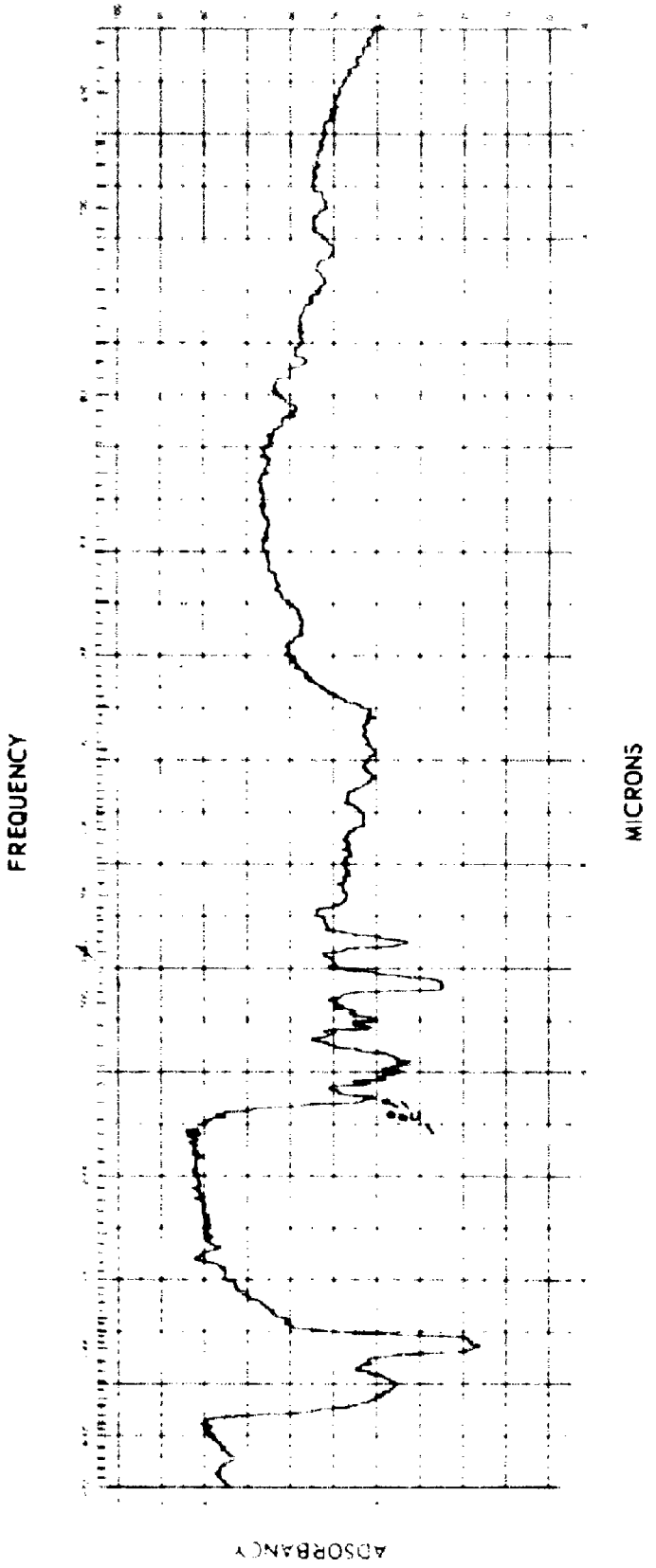

Figure 23. Infrared Absorption [illegible]

Extracted by [illegible] with [illegible] with methylether dried and analyzed by [illegible]

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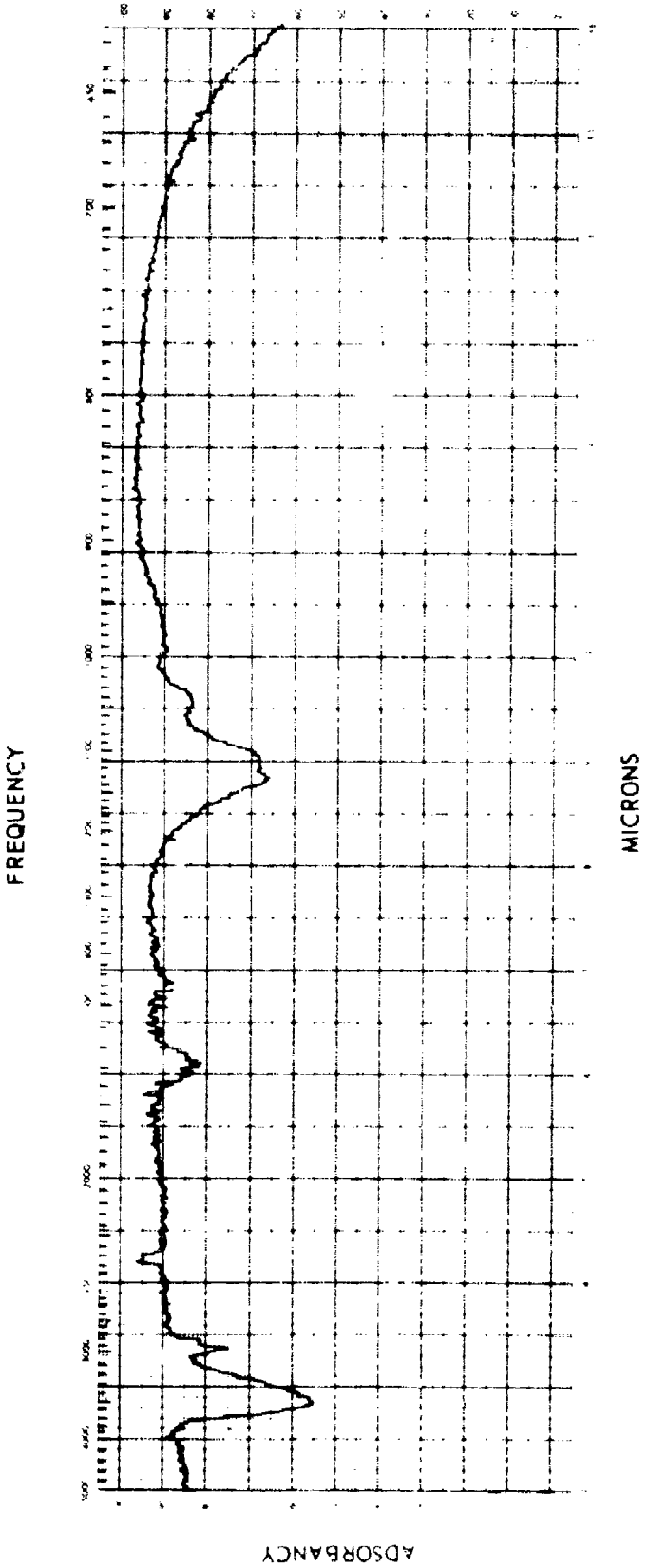


Figure 24. Infrared Absorption of Unnoculated Control

Extracted from Aqueous air with no foam with diethylether dried and analyzed by smear technique

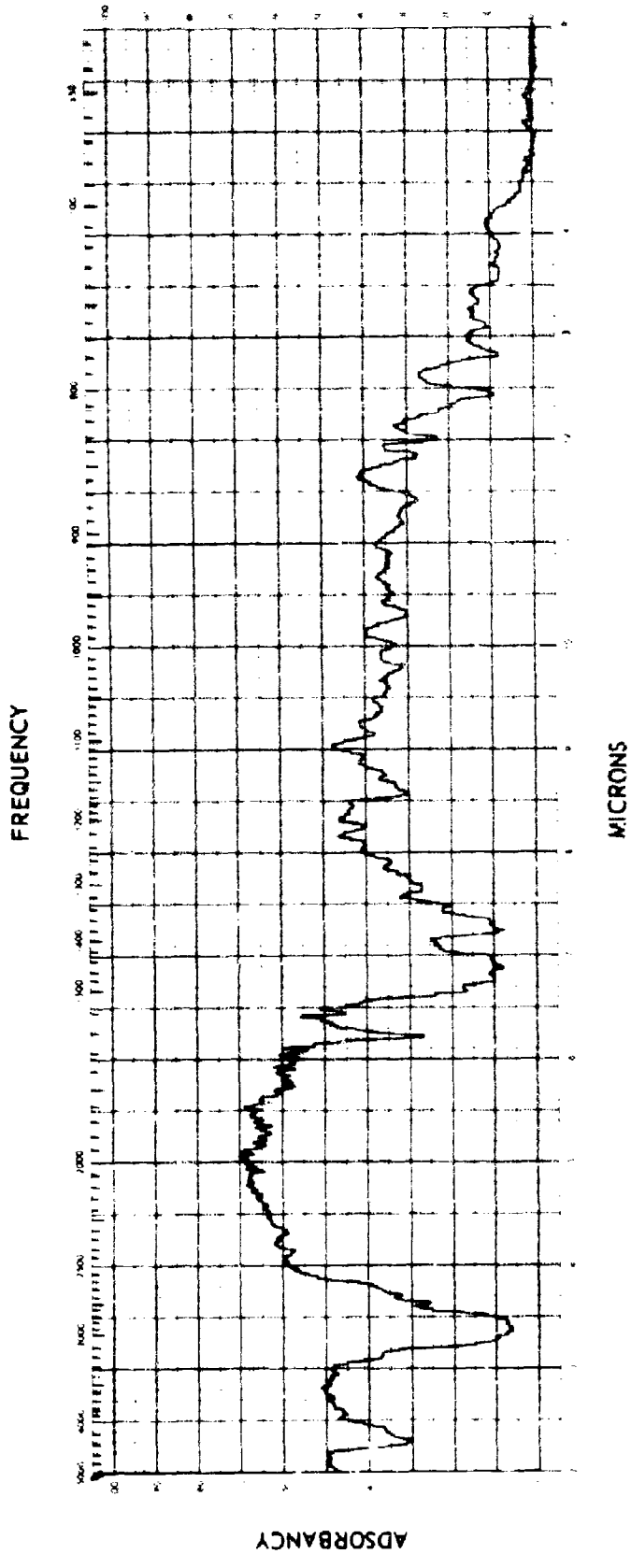


Figure 25. Infrared Absorption of Culture 89 Emulsion

Extracted from aqueous growth medium with diethylether dried and analyzed by smear technique

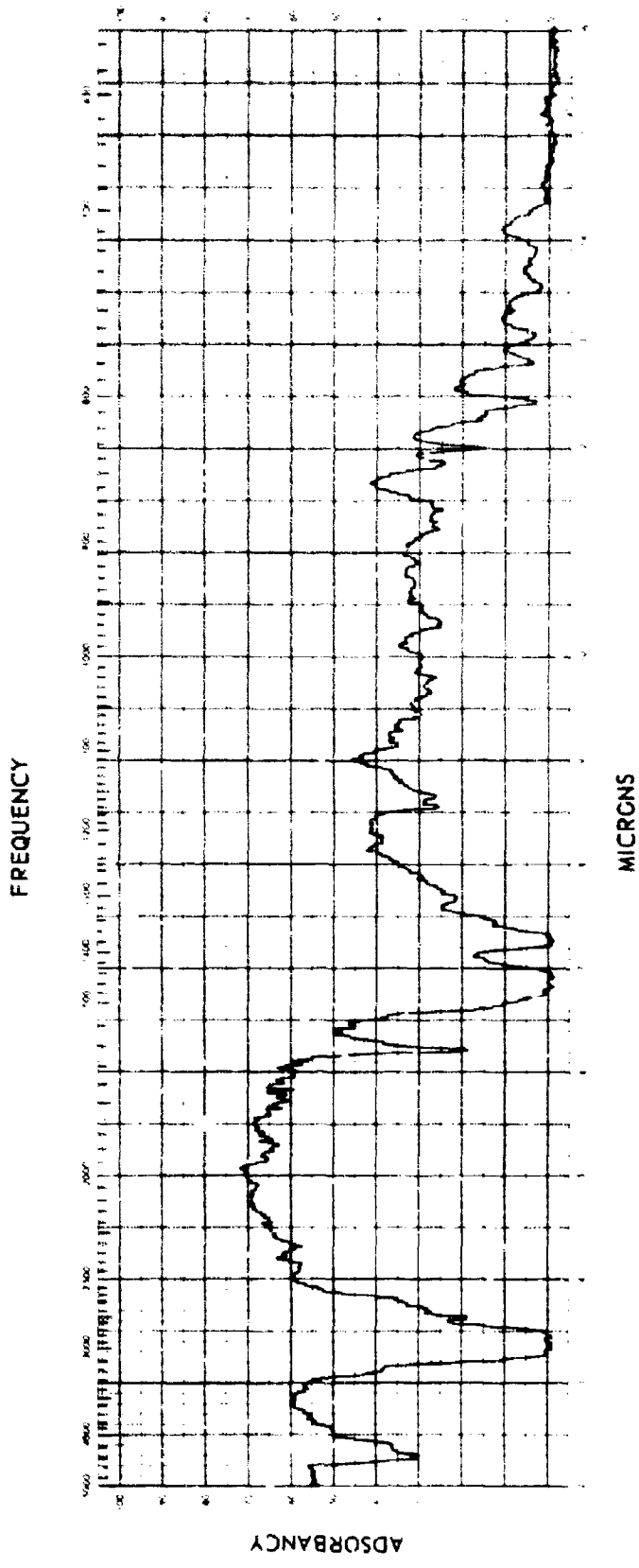


Figure 26. Infrared Absorption of Culture 94

Extracted from aqueous growth medium with diethylether dried and analyzed by smear technique

Future work will include the growth of culture 96 on pure aliphatic and aromatic hydrocarbons to determine if emulsion formation occurs. Attempts will also be made to get emulsion formation on the low mineral medium in JP-4 fuel, and on pure hydrocarbons. The correlation between corrosion and emulsion formation will then be made.

E. Nutritional Studies

In the past the most desirable medium for study of the corrosion mechanism was considered to be a Bushnell-Haas or BH medium. However, the corrosion experiments have shown that careful consideration has to be given to medium used in corrosion studies. For this reason two media, one containing copper, were selected from the nutritional studies at Sharpley Laboratories for investigation during this quarter. The methods and procedures for this study are described below.

1. Comparison of Growth and Corrosion on Medium A and BH

The comparison of growth with corrosion on Bushnell-Haas minus-nitrate fuel, medium A-fuel and modified A-fuel (see appendix) was carried out with isolate 96, originally obtained from fuel tanks. The sterile medium (100 ml overlaid with 20 ml JP4) was inoculated with 5 mls of culture. Controls of each medium were uninoculated.

Test and control cultures were incubated at 30°C on an incubator shaker for two days. Samples were withdrawn from test and controls at 0, 24 and 48 hours for determining viable counts. The results in Figure 27 show that medium A was better than BH or medium A containing sulfate instead of nitrate. The aluminum in each case was darkened and no pitting was observed. Further study of these media in addition to BH-nitrate media is planned during the next quarter to determine if differences in corrosion can be observed.

2. Effect of Copper

Preliminary investigation of the effect of copper on growth of three bacterial cultures was conducted by growing these cultures in medium A - nitrate fuel and medium A- nitrate fuel plus 0.03 grams of copper per liter. Investigations by Sharpley Laboratories showed that copper suppressed growth during the first few days but after long incubation periods a growth acceleration effect was noted.

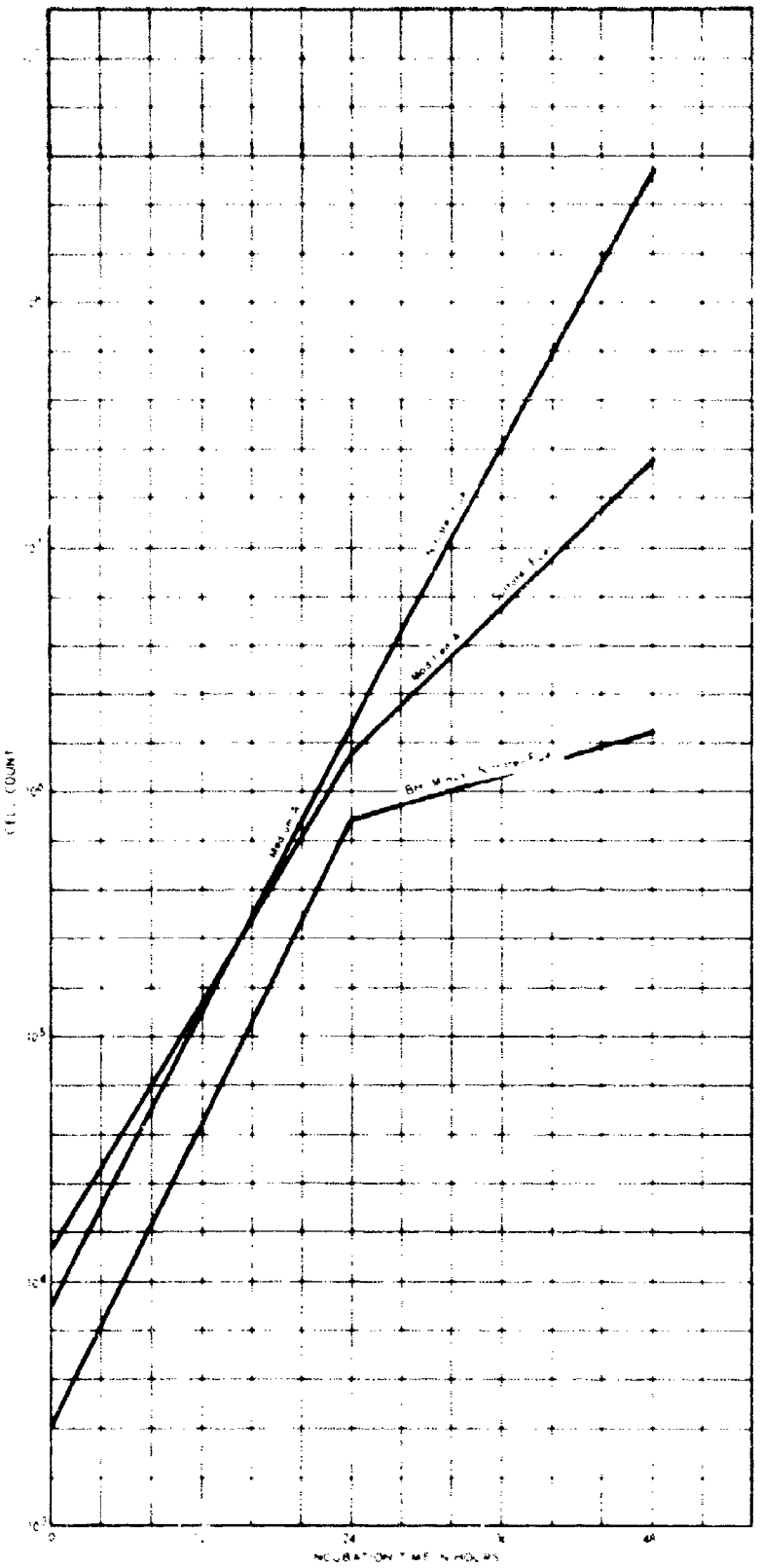
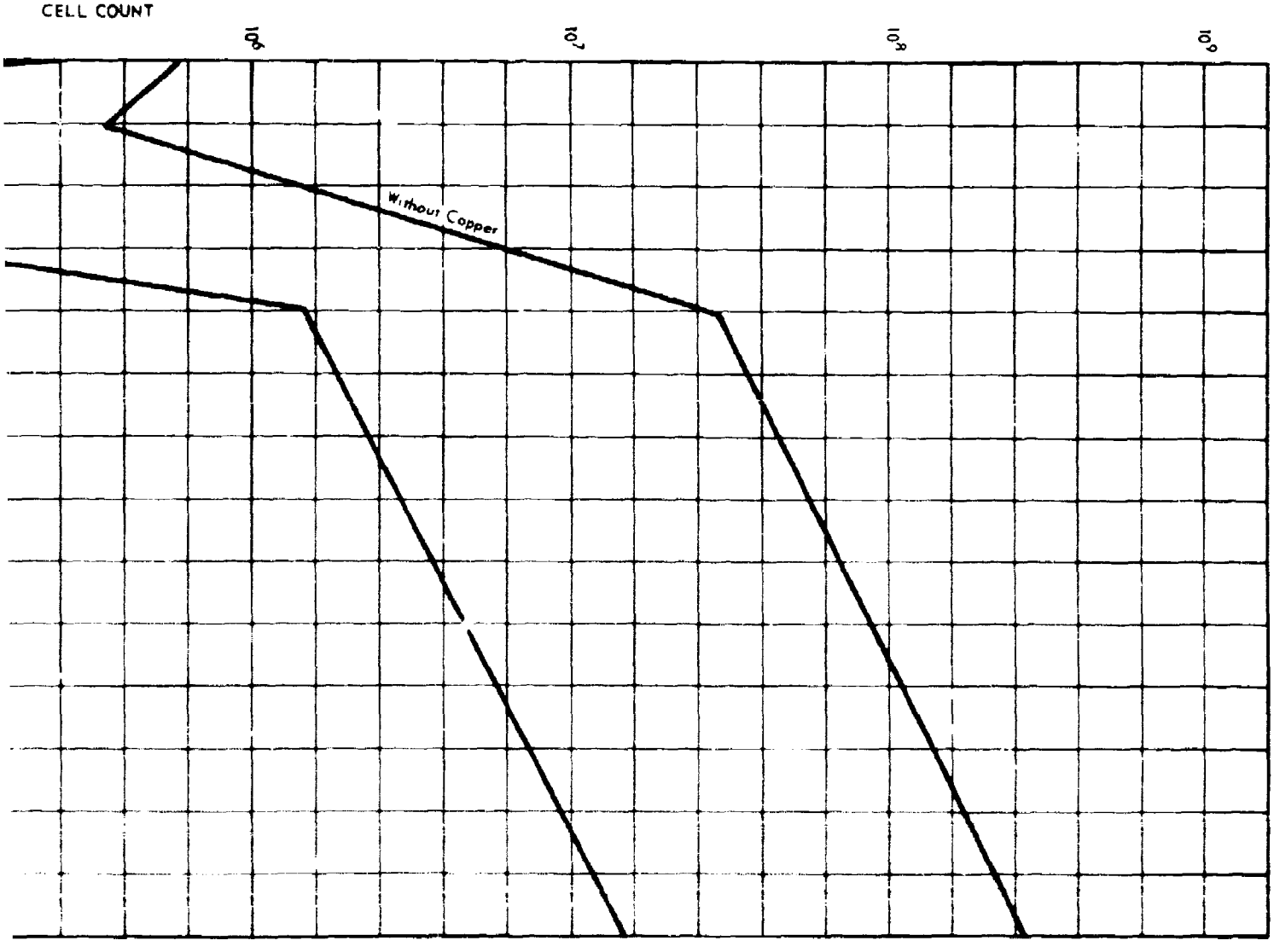


Figure 27. Mean Growth of Culture 96 in Three Media

Sterile Medium A nitrate-fuel plus copper and Medium A-nitrate fuel minus copper (100 ml, containing 7075 and 2024 alloys) were inoculated with five ml of cultures 89, 94, and 96. Test and control (uninoculated) flasks were incubated on a New Brunswick shaker at 30°C for 14 days. Growth occurring in these flasks was followed by viable plate counts in TGY agar after 0, 1, 4, and 14 days incubation. Less growth occurred with cultures of 89 and 94. The results indicated that all the cultures decreased in growth during the first day incubation in the presence of copper (Figures 28, 29, 30). Cultures 89 and 94 however, showed less growth in the presence of copper than in its absence during the first day whereas culture 96 showed greater growth in the first day in the presence of copper. After two weeks growth, however, there were no significant differences in growth between the two media.


CELL COUNT
10^6
10^7
10^8
10^9
Without Copper

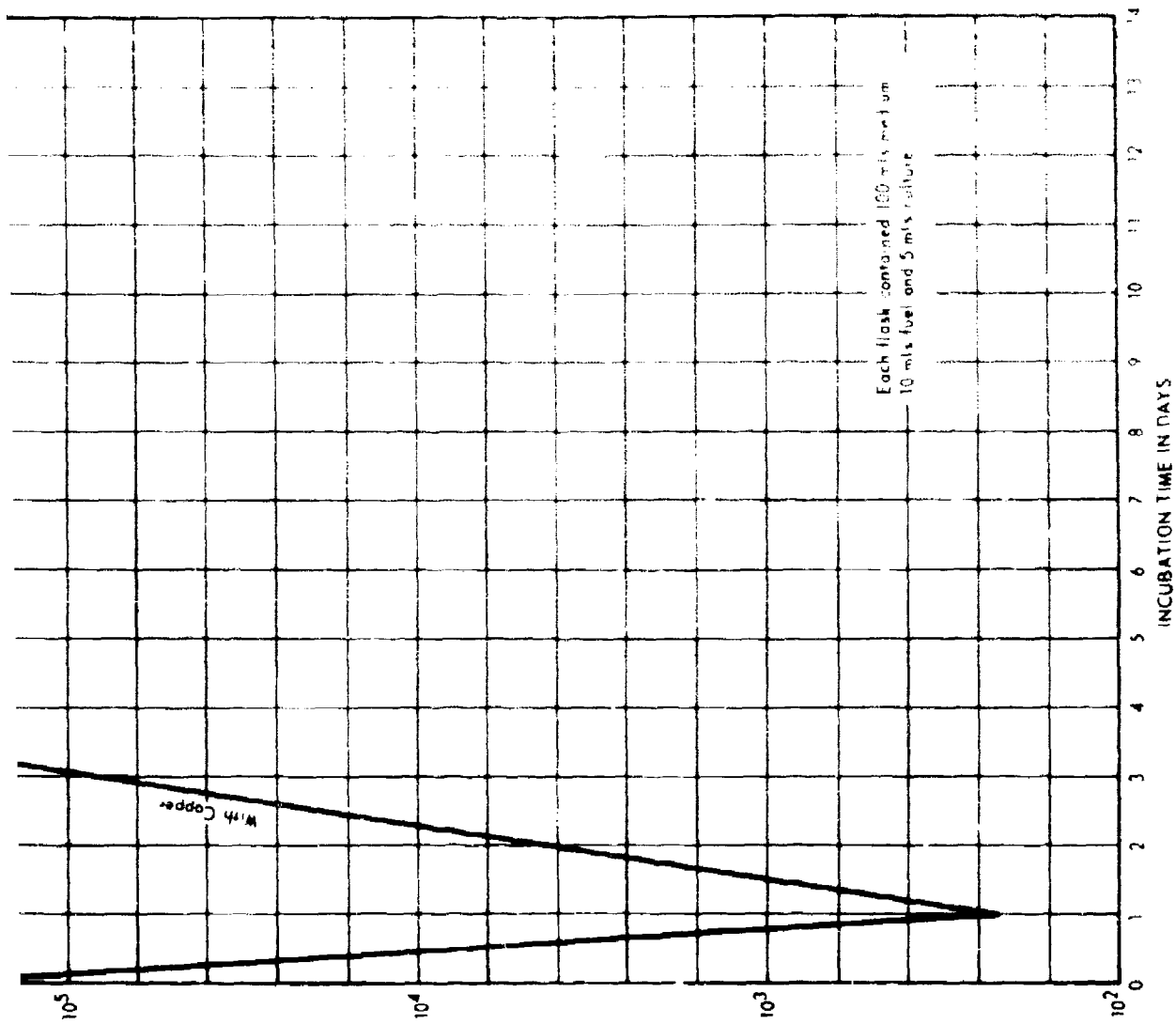


Figure 28. Mean Growth of Culture 89 in Medium A - Fuel Plus Copper, and Medium A - Fuel Minus Copper

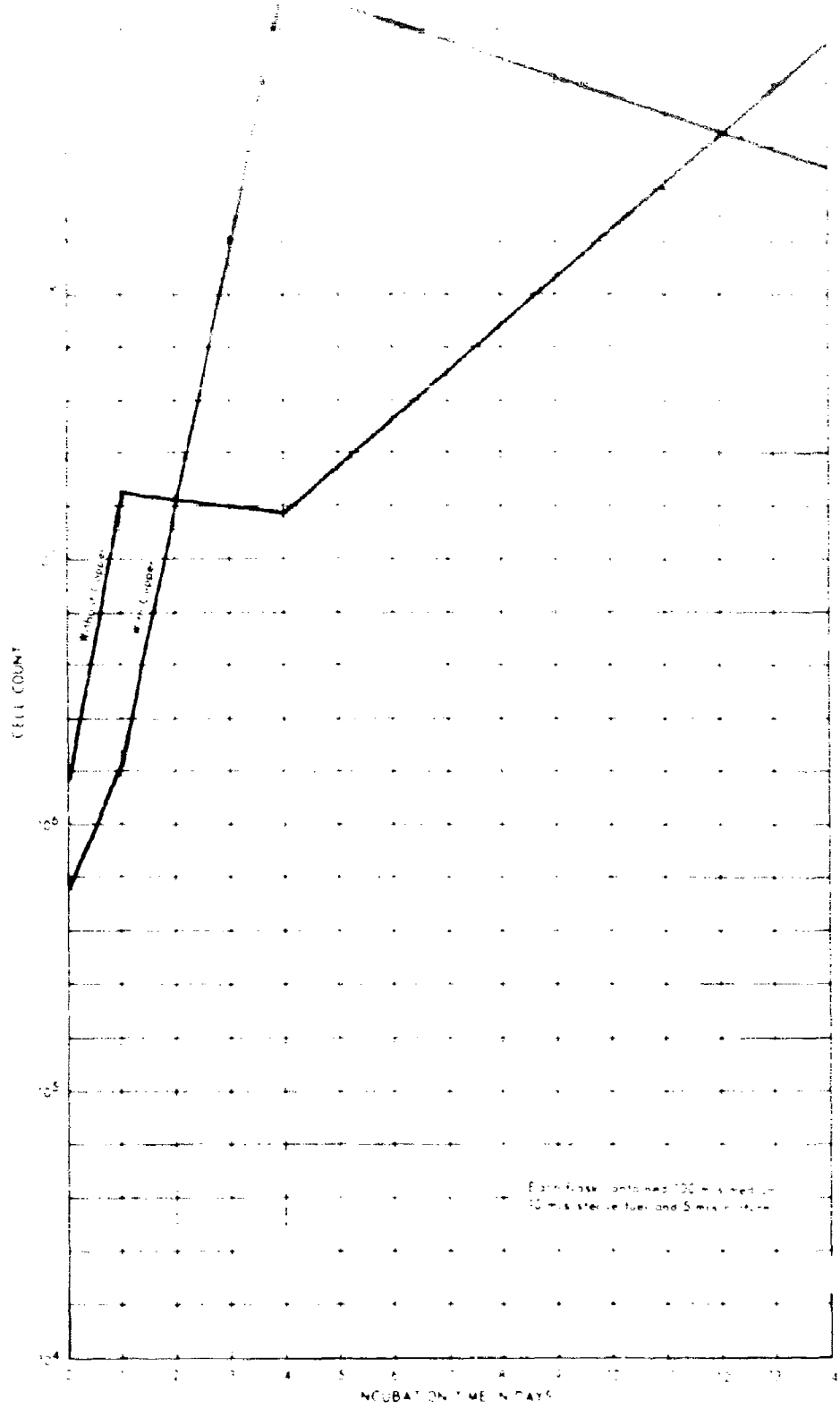


Figure 29. Mean Growth of Culture 94 in Medium A - Fuel Plus Copper and Medium A - Fuel Minus Copper

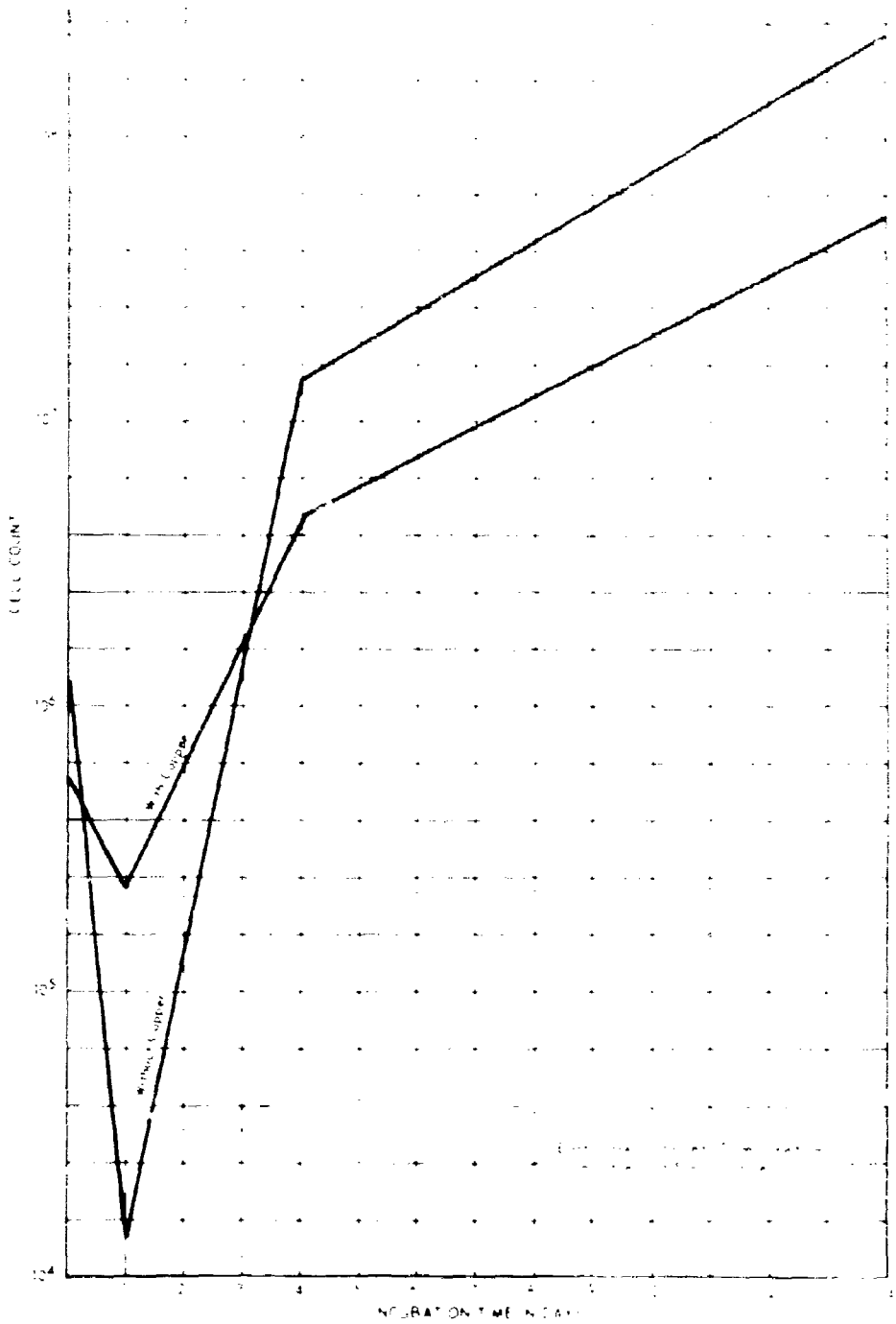


Figure 30. Mean Growth of Culture 96 in Medium A - Fuel Plus Copper and Medium A - Fuel Minus Copper

[illegible] of differences in cell membrane permeability to fuel components between normal cultures and fuel isolates. In one experiment, both normal and fuel isolates were grown in rich medium, washed and suspended in saline. The results showed that when the saline cultures were incubated under fuel all cells were killed. A second study utilizing growing cells in rich medium and mid-medium with fuel overlay showed that all cultures grew. Therefore, in the presence of rich medium fuel is definitely not lethal. In a third experiment, normal and fuel cultures were placed in BH-fuel medium. In this case the normal cultures were killed and fuel isolates survived and grew.

During the third quarter, resting cells of fuel isolates grown in BH nitrate fuel medium have been incubated under JP-4 fuel to determine if fuel grown cells are killed under conditions where they cannot grow.

To determine whether survival of fuel isolates in BH medium was due to growth, a medium was prepared which lacked a nitrogen source; sodium sulfate was substituted for ammonium sulfate in the BH medium. The experiment was conducted by inoculating five flasks containing 100 mls of BH-nitrogen free-fuel medium with 5 mls of test culture. Flasks of BH minus nitrate fuel medium inoculated with each of the five cultures served as controls. One ml samples were withdrawn from test and control flasks at 0, 24 and 48 hours incubation for viable plate counts.

The results presented in Table 14 indicate that growth occurred in all cultures, in both BH and BH-nitrate free medium. Careful examination of the reagents used in the modified BH medium revealed that 0.02 percent nitrogen was added to the medium through use of reagent grade chemicals. This small

TABLE 14

[illegible] IN BH-FUEL MEDIUM AND BH-NITROGEN FREE-FUEL MEDIUM*

[illegible]	pH	BH-nitrogen free 8	BH 17	BH-nitrogen free 17	BH 27	BH-nitrogen free 27	BH 96	BH-nitrogen free 96
[illegible]	[illegible]	1.1x10^5	3.2x10^5	8.3x10^5	2.4x10^4	5.[illegible]x10^4	9.6x10^4	1.[illegible]
[illegible]	[illegible]	1.[illegible]	3.8x10^5	1.0x10^6	2.1x10^4	7.[illegible]10^4	9.[illegible]x10^4	1.[illegible]x10^5
[illegible]	[illegible]	[illegible]	[illegible]	1.5x10^7	1x10^6	3.[illegible]x10^7	1.2x10^6	[illegible]
[illegible]	[illegible]	7.[illegible]x10^5	[illegible].3x10^5	1.4x10^7	1.1x10^6	2.[illegible]x10^7	1.[illegible]	[illegible]x10^5
[illegible]	[illegible]	1.[illegible]x10^6	2.[illegible]x10^6	1.7x10^7	[illegible].[illegible]x10^6	3.0x10^7	[illegible]x10^5	[illegible]x10^5
[illegible]	[illegible]	1.2x10^6	2.[illegible]x10^6	1.7x10^7	3.[illegible]x10^6	3.1x10^7	[illegible]x10^5	[illegible]x10^5

[illegible] Lab-Line Orbital Shaker at 30°C. Each flask contained 5 mls culture, 10 mls medium and [illegible]. [illegible] was done in TGY agar.

[illegible] after 24 hours growth.

amount of contamination appears sufficient to support growth. Further attempts to obtain nitrogen free salt solutions are planned for the next quarter. One approach might be to grow the cells in the BH-nitrogen-free fuel medium then centrifuge them out, resterilize the medium and test it again for ability to support growth. Unless the cells are fixing nitrogen it would be removed by the cells during growth and thus a nitrogen free solution could be obtained. Another approach might be the growth of the cells in rich medium and BH-fuel medium, followed by washing and resuspension in distilled water. The amount of killing or growth of these cells when exposed to a distilled water-fuel environment could be followed by determining viable cell counts during the incubation period. Also to be studied in the next quarter will be the effect of saline and fuel on resting cells of fuel grown organisms.

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APPENDIX

Laboratory Media

1. Medium A-Nitrate Fuel

		Gram per liter
Mg. $SO_4 \cdot 7H_2O$		0.4
$CaCl_2$		0.02
$K H_2 PO_4$		2.0
$K_2 H PO_4$		2.0
$NH_4 NO_3$		2.0
Fe Powder		4.0
Distilled H_2O	to	1000 mls
Aluminum coupon		
Fuel**		20 mls/100 cc
pH 7.0		

** Sterilize fuel by Millipore filtration and add aseptically

2. Medium A-Nitrate-Fuel plus Copper add 0.039 grams of $CuSO_4 \cdot 5H_2O$ to medium A-nitrate fuel

3. Medium A-Sulfate - fuel
Substitute $(NH_4)_2SO_4$ for NH_4NO_3 in same quantities.

4. Formula F: (8)

NH_4Cl		1.0g
$MgSO_4$		0.1g
KH_2PO_4		1.5g
Na_2HPO_4		3.5g
Lactic Acid***		9.0g
Distilled H_2O	to	1000 mls
Adjust pH to 7.3 with NaOH		

*** NA Lactate, 10.0g, may be substituted here.

5. Bushnell-Haas Salts Soln.

$MgSO_4$		0.2g
KH_2PO_4		1.0g
K_2HPO_4		1.0g
$(NH_4)_2SO_4$		1.0g
$CaCl_2$		0.02g
$FeCl_3$		0.05g
Distilled H_2O	to	1000ml.

Adjust pH to 7.2 with NaOH or HCl

6. TGY Agar

Tryptone		5.0 grams
Yeast Extract		5.0 grams
Glucose		1.0 grams
K_2HPO_4		1.0 grams
Agar		20.0 grams
Tap H_2O	to	1000 ml

pH 7.0

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